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PRACTICAL MICROSCOPY

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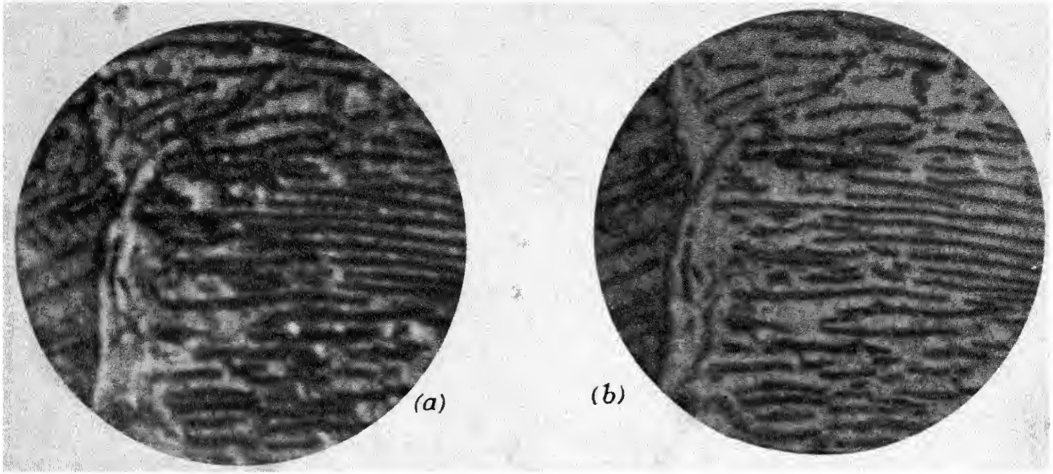

COMPARISON PHOTOMICROGRAPHS (taken in visual and ultra-violet illumination) showing increased resolution due to use of shorter wave-length light. The specimen is a 0·8 % carbon steel.

(*a*) Objective:—2 mm. Apochromat.
Numerical Aperture = 1·30
Magnification = 4000 ×
Wave-length of Light = 0·55μ.

(*b*) Objective:—1·7 mm. Monochromat.
Numerical Aperture = 1·25
Magnification = 4000 ×
Wave-length of Light = 0·275μ.

Frontispiece

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PRACTICAL MICROSCOPY

BY

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BLACKIE & SON LIMITED
LONDON AND GLASGOW
1931

Printed in Great Britain by Blackie & Son, Ltd., Glasgow

PREFACE

The writers have attempted to bring together in these pages the information necessary to apply in practice the technique of modern microscopy. For the proper understanding of the use of the microscope, a knowledge of the optical principles and physical limitations involved is essential; and it has been felt that if these were put down in simple and concise form, it would help the microscopist to use his instrument to better effect. The book is intended to be of a practical nature, as a perusal of the contents page will show; and in order that it shall include the most recent work a chapter on ultra-violet microscopy also appears.

We wish to make acknowledgment and express our thanks to the following firms, who have rendered us considerable help by supplying blocks for many of the illustrations: Messrs. Baker, Busch, Cambridge Instrument Co., Leitz, Swift, Watson, and Zeiss.

It is hoped that these pages may be of help to all those interested in microscopy in any of its forms.

L. C. MARTIN.
B. K. JOHNSON.

ROYAL COLLEGE OF SCIENCE,
October, 1931.

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PRACTICAL MICROSCOPY

CHAPTER I

Magnification

The Apparent Size of an Object

The size of the image of a given object formed on the retina of the eye is determined by the angle which that object subtends at the optical centre (or nodal point) of the eye.

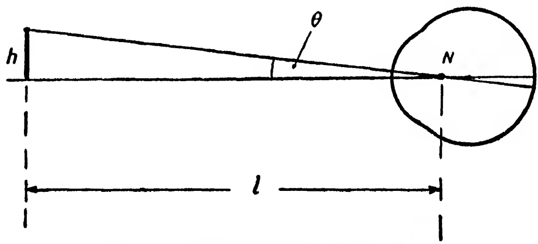


Fig. 1.—Apparent Size of an Object

Thus in fig. 1 the apparent size of the object h is represented by the *visual angle* θ, and can be measured by expressing it in the term $\frac{h}{l}$, which is the tangent of θ, i.e.,

$$\tan\theta = \frac{h}{l}. \qquad (1)$$

In order, therefore, to see an object as large as possible with the unaided eye it is necessary to place the object as near

to the eye as possible compatible with distinct vision, i.e. at the "near point". This least distance of distinct vision is now conventionally given as 10 in. or 250 mm. for normal sight and is frequently denoted as D_v, so that, if our object h (in fig. 1) was placed at the near point of the eye, its visual angle would now be

$$\text{Visual angle (measured by tangent)} = \frac{h}{D_v}. \quad . \quad (2)$$

Magnification by an Optical Instrument

Those optical instruments which are used for magnifying an object are pieces of apparatus by means of which the size of the retinal image of an object is increased, and the ratio of the size of this image to that of the object when seen with the eye alone is termed the magnifying power of the instrument. This may be written:

$$\text{Magnifying power of instrument}$$
$$= \frac{\text{size of retinal image of object seen with instrument}}{\text{size of retinal image of object seen with unaided eye}}$$
$$= \frac{\text{visual angle of image seen with instrument}}{\text{visual angle of object seen directly}},$$

the angles being measured by their tangents, as above.

Principle of Hand Magnifier or Simple Microscope

If a small object is placed in the focal plane of a short-focus lens which is held in front of the eye, a magnified virtual image of the object is seen. This image is seen apparently at an infinite distance. The principle will be clear from fig. 2, from which it will be noticed that parallel bundles of rays leave the lens to enter the eye; the inclination θ of these rays to the axis is dependent on h and f, and therefore the visual angle of the image is now $\frac{h}{f}$.

Thus the magnification by a simple lens =

$$\frac{\text{visual angle of image seen with lens}}{\text{visual angle of object seen at near point with unaided eye}}$$

$$= \frac{\frac{h}{f}}{\frac{h}{D_v}} = \frac{D_v}{f} \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

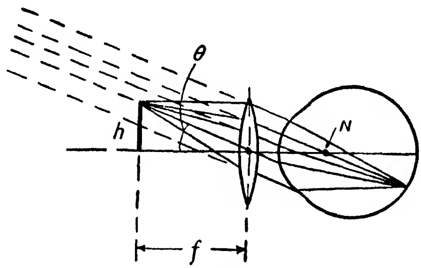


Fig. 2.—Object viewed with a Short-focus Lens

This means that the focal length of the magnifier (expressed in inches or millimetres) is divided into 10 inches or 250 mm. respectively. The resulting figure gives the magnifying power.

For example:—

An eyepiece of 1″ focal length has a magnifying power

$$= \frac{10}{1} = \times 10.$$

An eyepiece of 50 mm. focal length has a magnifying power

$$= \frac{250}{50} = \times 5.$$

There are many and varied types of "simple microscope" to be obtained. First, there is the ordinary hand magnifier mounted in a suitable handle, and intended for general observations on almost any kind of object; many of these still consist of single lenses, although most modern types are corrected for chromatic and spherical aberration and are expected to have a flat field. An example of the latter so-called "Steinheil" type is shown in fig. 3, and consists of a double-convex crown lens cemented between meniscus

lenses of flint. Such a lens may be mounted on a suitable stand or immediately above a microscope stage and used in dissecting work (see fig. 4).

A further application is to mount a glass scale (usually 10 mm. divided into 100 parts) in the focal plane of the magnifier, so that the scale lies in the same plane as the object, which is illuminated by a plane glass reflector. By means of such a device (fig. 5) it is possible to take measurements of the relative size of objects under observation. The so-called scaleometer (made by Messrs. Ottway) is an example of this instrument.

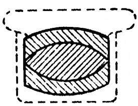


Fig. 3. Hand Magnifier (Achromatic Type).

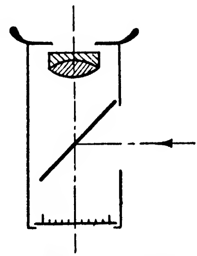


Fig. 5.—Eyepiece with Scale mounted in Focal Plane.

Focal Length of Eyepieces

In order to determine the power of a simple magnifier, or in fact any compound eyepiece, it will be seen from the

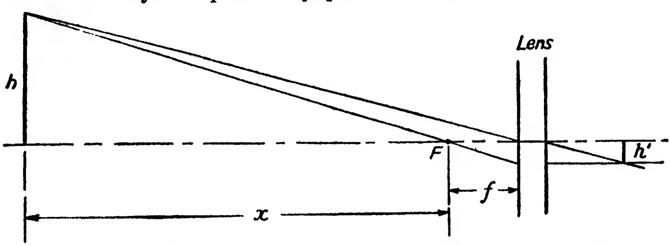


Fig. 6.—Method of measuring Focal Lengths of Eyepieces

foregoing relation $M = \frac{D_v}{f}$ that a knowledge of its focal length is necessary. The following method may be used for this determination.

An object (consisting of a piece of white card 3 ft. long held against a dark background) is set up at a distance of about 10 ft. from the eyepiece. The reduced image h' (fig. 6)

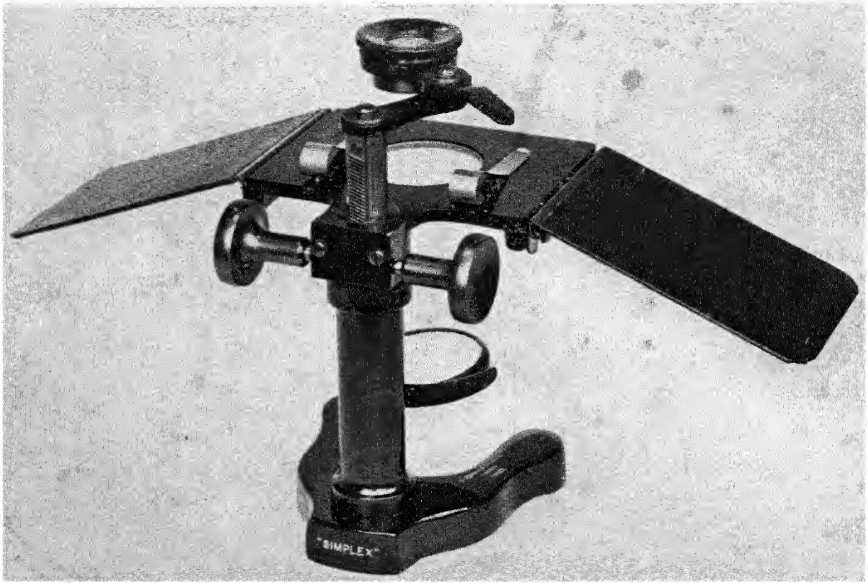


Fig. 4.—SIMPLE FORM OF DISSECTING MICROSCOPE (By *Watson*)

of this is observed and measured by means of an auxiliary eyepiece having a glass scale (divided into tenths of a millimetre) mounted in its focal plane. By measuring up the distance x, the three quantities h, h', and x will be known, from which the focal length f can be found; for from the figure $\frac{f}{h'} = \frac{x}{h}$.

Actually, the distance x is that between the first principal focus of the lens under test and the object, but in practice if this distance is large as compared with the focal length of the eyepiece, the distance may be measured from the object to the lens without introducing any serious error. It should be noted that x and h must both be measured in the same units (say in feet), and also h' and f in the same units as each other although not necessarily in those used for x and h; for instance, h' and f may be in inches or millimetres. Thus:

Focal length in inches

$$= \frac{(\text{size of image in inches}) \times (\text{distance of object in feet})}{(\text{size of object in feet})}.$$

In using this method for an eyepiece, turn the eye-lens towards the object.

Focometers

There are complete instruments, known as focometers, for the quick determination of focal lengths of eyepieces and incidentally of microscope objectives. Such an instrument is shown diagrammatically in fig. 7; this type is due to Professor F. J. Cheshire.*

It consists of a small collimator (with two vertical lines A and B) suitably mounted in front of the lens to be tested, the image A′B′ produced by the latter (on account of its small size) being examined by a microscope. From the diagram it will be evident that $f_e = \frac{f_c}{AB} \cdot A'B'$, where f_c and f_e are the

* *Journ. Roy. Micro. Soc.*, 1914, pp. 513–9.

focal lengths of collimator lens and test lens respectively.

If the observing microscope has a magnification equal to M, and the size of the final image on the scaled eyepiece of the microscope is A″B″, then $\frac{A''B''}{M} = A'B' = \frac{f_e}{f_c} . AB$, and therefore,

$$f_e = A''B'' . \frac{f_c}{M . AB}. \quad . \quad . \quad . \quad . \quad (4)$$

The value $\frac{f_c}{M . AB}$ will be a constant for any one instrument, and therefore the determination of the focal length of the lens system under test merely becomes an operation

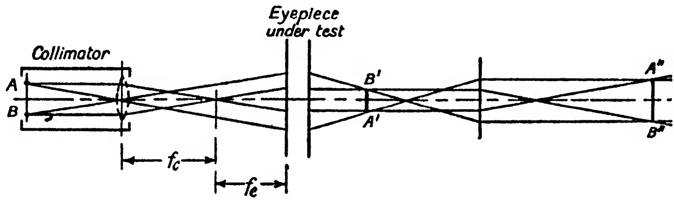


Fig. 7.—Principle of Focometer

of measuring A″B″ and multiplying it by a predetermined factor. It is not difficult by adjusting the dimensions of AB and the value of M to make this factor say 10, whence the instrument becomes a direct reading one.

Magnification of Compound Microscope

The formation of the image seen with the compound microscope will be understood from the ray diagram of fig. 8. The objective projects an enlarged and inverted image of the object, thus giving a primary (linear) magnification of

$$\frac{h'}{h} = \frac{g}{f_o} \text{ so that } h' = \frac{h \times g}{f_o}. \quad . \quad . \quad . \quad . \quad (5)$$

The distance g between the adjacent focal planes of the

objective and eyepiece is known as the *optical tube length.* Thus the " primary magnification ",

$$\frac{h'}{h} = \frac{\text{(optical tube length)}}{\text{(focal length of objective)}}.$$

The image h' is viewed by an eyepiece of focal length, say f_e, and the resultant virtual image is seen apparently at an infinite distance. The angle (measured by its tangent) under which this latter image will be seen is $\frac{h'}{f_e}$, which from (5) gives

$$\frac{h \times g}{f_o \times f_e}.$$

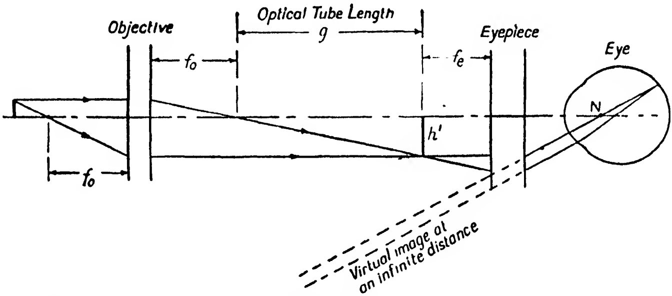


Fig. 8.—Ray Diagram showing Formation of Image in the Compound Microscope

But if the object were viewed by the unaided eye at the normal distance of distinct vision D_v, it would be seen under the angle whose tangent is $\frac{h}{D_v}$. Hence,

Magnification of compound microscope

$$= \frac{\text{(visual angle of image seen with instrument)}}{\text{(visual angle of object seen with unaided eye)}} = \frac{\dfrac{h \times g}{f_o \times f_e}}{\dfrac{h}{D_v}}$$

$$= \frac{g \times D_v}{f_o \times f_e}. \quad . \quad . \quad . \quad . \quad . \quad . \quad (6)$$

Alternatively the total magnifying power of the microscope may be expressed as the primary magnification multiplied by the power of the eyepiece, which is the equivalent of the formula (6), namely,

$$\left(\frac{g}{f_o}\right) \times \left(\frac{D_v}{f_e}\right).$$

These two individual quantities (in brackets) are the ones usually employed in the catalogues of English microscope makers for designating the magnifications of the objectives and eyepieces listed, but continental makers frequently give the objective magnification as $\left(\frac{D_v}{f_o}\right)$ and the eyepiece power as $\left(\frac{g}{f_e}\right)$.

An example will illustrate the two systems:

Take an objective of 4 mm. focal length, an eyepiece of 10 mm. focus, and assume an optical tube length of 160 mm.

Then, by English system,

$$M = \frac{g}{f_o} \times \frac{D_v}{f_e} = \frac{160}{4} \times \frac{250}{10} = 40 \times 25 = \underline{1000},$$

and by continental system,

$$M = \frac{g}{f_e} \times \frac{D_v}{f_o} = \frac{160}{10} \times \frac{250}{4} = 16 \times 62{\cdot}5 = \underline{1000}.$$

In the English system, the magnification of the 4 mm. objective is 40, and that of the 10 mm. eyepiece is 25; but in the continental system, the magnification of the objective is 62·5 and that of the eyepiece is 16.

In cases of ordinary visual observation the magnification need seldom be known to any great order of accuracy; it usually suffices to take the values given in the makers' catalogues or to rely on the focal lengths of the objectives and the power of the eyepiece (both of which are generally engraved on these parts) and use these in the formula given in the above example to work out the value. Table I gives a list of magnifying powers, the magnifications of the eyepiece being given in the English system.

TABLE I

Objective.	Primary Magnification (Tube length 160 mm.).	Total Magnifying Power. Eyepiece.					Working distance (appr.).
		×6	×10	×15	×20	×25	mm.
2″ or 50 mm.	3·2	19	32	48	64	80	30
1″ or 25 mm.	6·4	38	64	96	128	160	14
$\frac{2}{3}$″ or 16 mm.	10	60	100	150	200	250	8
$\frac{1}{3}$″ or 8 mm.	20	120	200	300	400	500	2
$\frac{1}{6}$″ or 4 mm.	40	240	400	600	800	1000	0·5
$\frac{1}{8}$″ or 3 mm.	53	318	530	795	1060	1325	0·3
$\frac{1}{12}$″ or 2 mm.	80	480	800	1200	1600	2000	0·2

There are cases, however, when such figures may not be sufficiently accurate for the particular work on hand, and a direct determination of the magnification will have to be resorted to.

Direct Measurement of Magnification by Visual Observation

A " stage micrometer " (i.e. a 3 in. × 1 in. slide with object consisting of a series of ruled lines spaced at intervals of 1 mm., $\frac{1}{10}$ mm., and $\frac{1}{100}$ mm. —see fig. 9) is placed on the microscope, illuminated and focussed. The latter is swung into a horizontal position and raised if necessary until the axis of the microscope is 23 cm. from the table (see fig. 10). A microscope cover glass is then mounted over the eyepiece at 45° to the axis, so that by this means the eye, when looking downwards, can see the image of the object lines apparently on a piece of white paper pinned to the table. By choosing two lines suitably spaced their positions can be marked with a pencil and their separation

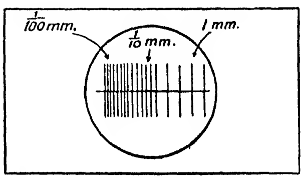


Fig. 9.—Stage Micrometer

measured up. If the distance apart of these two marks is p, whilst the original object size is h, then

$$\text{Magnification} = \frac{\dfrac{p}{D_v}}{\dfrac{h}{D_v}} = \frac{p}{h}.$$

See also method given in section on Numerical Aperture.

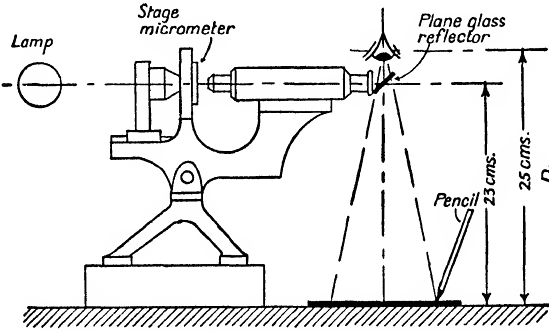


Fig. 10.—Direct Measurement of Magnifying Power

Use of Microscope as a Measuring Instrument

When it is desired to ascertain the actual size of some small object—for example, the dimensions of a bacterium—it becomes important to know accurately the primary magnification of the microscope and also to have a means of measuring the size of the image. A piece of apparatus known as an "eyepiece micrometer scale" (shown in fig. 11) is usually employed for this purpose, and as its name implies, consists of a glass scale (divided into tenths or twentieths of a millimetre) mounted in the focal plane of the eyepiece.

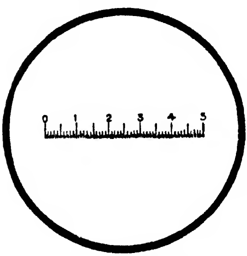


Fig. 11.—Eyepiece Micrometer Scale

By placing a stage micrometer on the microscope stage,

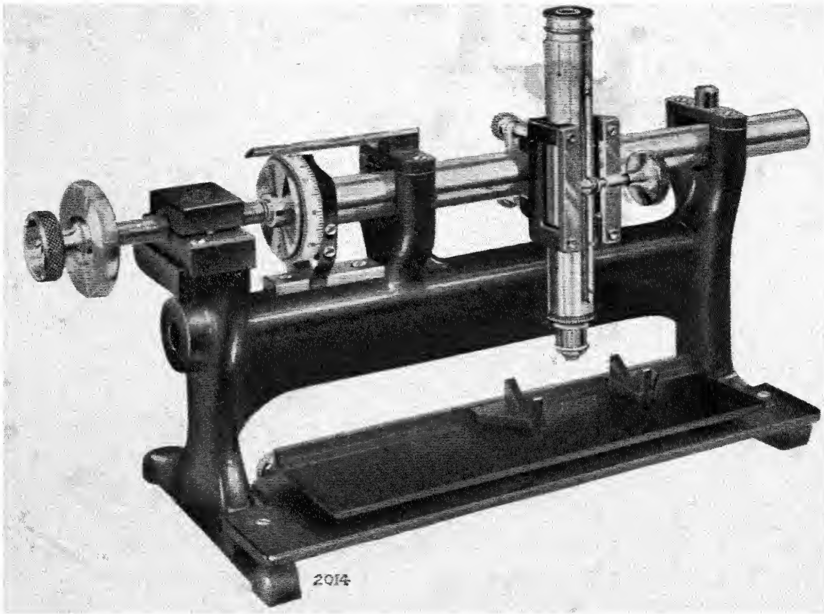


Fig. 12.—CAMBRIDGE MEASURING MICROSCOPE

The fine adjustment must be sufficiently well made to fulfil the conditions of providing a motion which will comply with the *focal depth* requirements of the highest power lens to be used on the instrument. In the case of the 2 mm. immersion objective this depth of focus (as shown on p. 39) is of the order of 0·4 microns.

The *stage* consists of a rigid platform on which the object to be examined is placed. It is frequently fitted with, or built in with, a *mechanical stage* which affords delicate movement of the object slide, the adjustment being made by the knurled heads on the right of the stage seen in fig. 13. The motions thus provided sometimes aid in the measurement of, and in the systematic search for, an object; more especially if the amount of travel of the stage is recorded in two directions by means of scales and vernier.

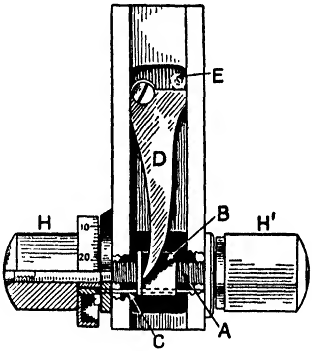


Fig. 14.—A Form of "Fine Adjustment" used on a Microscope

The *substage* is in effect the mount into which the condenser fits. It is attached to the limb in a suitable slide, and arrangements are made on it for the centring and focussing of the condenser. At the lower extremity of the substage is the *tailpiece* to which the mirror is attached. No substage condenser is necessary for use with low power objectives, say 1 in., and it is a convenience to be able to remove it from the path of the light. The condenser is fitted with an iris diaphragm, and there may be fittings for holding light-filters, i.e. blue or green glasses. In some cases, the iris can be given a lateral movement by a rack and pinion.

Whilst dealing with the subject of mechanical details, the objective mount is of some importance. The advantage

of being able to locate the object with a lower power objective prior to using the one desired need not be emphasized, but the want of some convenient means of changing quickly from one objective to the other is soon realized. To meet this need several types of objective changer were devised—one of these known as a "revolving nosepiece" consists of a rotary mount (suitably secured to the lower end of the body tube) in which there are three threaded apertures to receive the objectives. The latter can in turn be brought in line with the axis of the microscope at will by a simple rotation of the metal plate carrying the lenses; such a mount is depicted at K, fig. 13. Whilst this device may be quite satisfactory for powers up to a $\frac{1}{6}$ in., it frequently happens that when a $\frac{1}{12}$ in. is so mounted and swung into position the object which was previously set in the centre of the field no longer appears there at all. This lack of alignment is partly due to mechanical difficulties and partly to differences in centring of the objectives. To overcome this, devices have been made whereby each object glass can be properly centred by adjusting screws, and can be removed quickly and conveniently by a type of sliding mount. Such types by the firms of Zeiss and Leitz, and more recently one due to Professor A. F. C. Pollard* are amongst the more prominent examples of the interchangeability of objective mounts with precise alignment.

* An eyepiece and interchangeable nosepiece for "centring" and "squaring on" microscopic objectives.—*Proc. Opt. Convention,* 1926.

CHAPTER III

Objectives and Eyepieces

Objectives

The chief function of a microscope objective is to produce the primary magnified image which is in turn viewed by the eyepiece. That this image shall be merely magnified is scarcely a sufficient requirement; it must also be sharply defined. The earliest form of objective consisted of a simple short-focus lens, very often equi-convex, and made of one kind of glass only. It was found with such a lens that the image produced by it was surrounded by coloured fringes and that the details were fuzzy and indistinct. Attempts at correcting these two chief defects, namely, chromatic and spherical aberration (see fig. 15), were made in order to obtain a better image. The first of these was corrected by employing two different types of glass for the lens components and combining them so that the resultant dispersion was annulled or nearly so. By suitably arranging the shape of the lens or lenses, it is possible to reduce the spherical aberration to a minimum for given conditions; in addition to these there are two other aberrations, namely, astigmatism and coma, which have to be contended with, and it is found that only by employing several lenses of different glasses and shapes can a really well-defined image be obtained. A schematic illustration of typical microscope objectives of various powers is given in fig. 16.

The reason for microscope lens systems taking this particular form will be seen from the following brief treatment of the subject.

Figure 17 (*a*) represents a plano-convex doublet-achromatic lens, placed with its plane side nearer to the object; such a lens has the property that rays diverging from one special object point O on the axis will be brought together at O′

(i.e. there is freedom from spherical aberration). These points are said to be " aplanatic ". This shape of lens also has another pair of " aplanatic points " which is represented

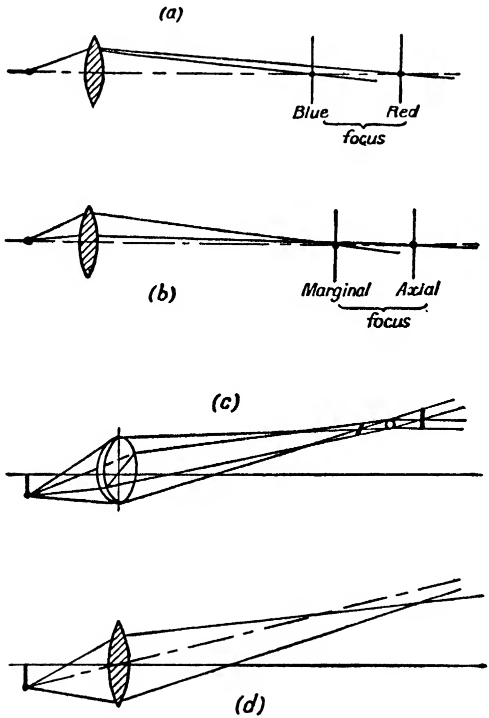


Fig. 15.—Forms of Lens Aberration (diagrammatic)
(*a*) Chromatic aberration. (*b*) Spherical aberration.
(*c*) Astigmatism. (*d*) Coma.

in fig. 17 (*b*); in this case, a cone of rays diverging from a certain point O (within the focus of the lens) is refracted as though the rays diverge from a second virtual point O′.

By extending the principle of fig. 17 (*b*), J. J. Lister, some 100 years ago, developed a higher power objective by

incorporating two achromatic lenses in train, in such a way that the first gave aplanatic refraction with a virtual image

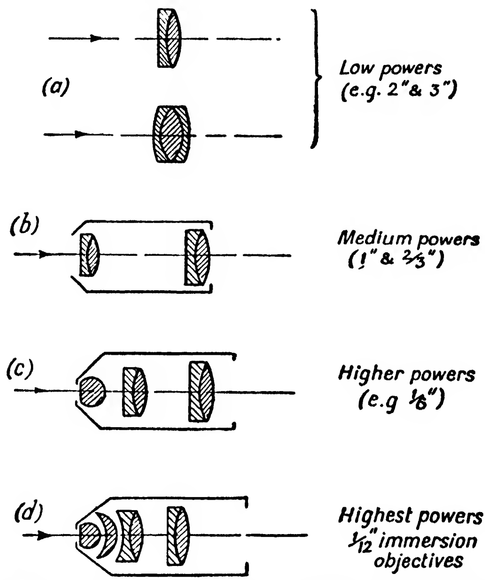


Fig. 16.—Optical Construction of Microscope Objectives

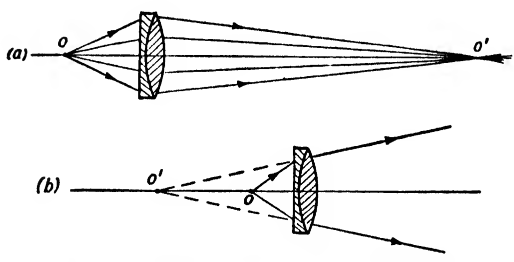


Fig. 17.—Aplanatic Points of a Lens System

at O′ (fig. 18), this point serving as one of the aplanatic points of the second lens, whilst the conjugate aplanatic point of this lens is at O″, the position of the real image.

A further step in the application of the aplanatic refraction principle enabled still higher powers and higher aperture objectives to be made.

Imagine a sphere of glass (denoted in thick line of fig. 19) with a refractive index n, and a radius equal to r. The dotted concentric circles represent spheres of radii nr and $\frac{r}{n}$; then

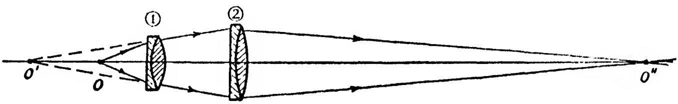


Fig. 18.—Lister Type of Objective

rays directed towards the point O on the outer sphere and refracted at the glass surface will all be directed towards a point O′ (on the same radius vector) on the surface of the inner sphere. Conversely, rays diverging from O′ will give a virtual image O, free from aberration.

On the above principle it is possible to avoid large spherical aberration by refraction at a spherical surface, while the rays are given a considerable con-

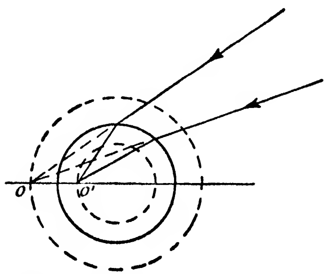


Fig. 19.—Refraction at a Spherical Surface

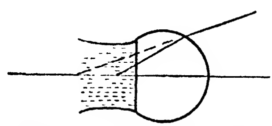


Fig. 20.—Front Lens of an Immersion Objective

vergence; thus the highest power objectives have a hemispherical or hyper-hemispherical front lens often immersed in a fluid of the same refractive index as the lens itself. With such an arrangement (depicted in fig. 20) there will be a certain amount of chromatic aberration produced; this has to be corrected by the back components of the lens system.

The complete optical system of these objectives is shown in fig. 16 (*c*) and (*d*).

Lenses up to $\frac{1}{6}$ in., or in some cases up to $\frac{1}{10}$ in., have air between the front lens and the cover-glass of the object and are known as " dry " lenses, whilst those just referred to are called " immersion " or sometimes " homogeneous immersion " lenses. The liquid used with the latter is special cedar-wood oil. (No other fluid should be used except when specially stated, such as in the case of water-immersion objectives.) Considerable care must be exercised in removing

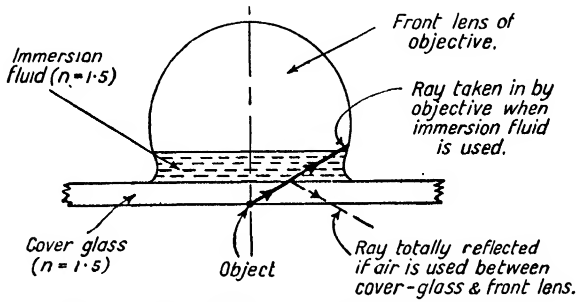


Fig. 21.—Illustrating Action of the Immersion Fluid

the " immersion oil " both from the objective and slide; this may be done by dipping a piece of silk in xylol or preferably benzene, and carefully wiping off the oil. As the hyper-hemispherical front lenses of such objectives are in many cases only held in their mount by means of a cement, care must be taken not to exert undue pressure on the lens when cleaning it, as this may result in displacing the lens from its seating.

A further purpose of the use of the immersion liquid is that it enables the objective to take in a larger cone of rays from the object than is otherwise possible (fig. 21 will make this clear); this is of considerable importance, for, as shown later, the larger the cone of rays taken in, the greater the resolving power obtainable.

Apochromatic Objectives

In the achromatic type of objective the colour correction is such that the red and blue rays are brought to a common focus, but they are not entirely free from secondary or residual spectrum. In a great many cases, however, this does not matter, and this type is consequently widely used. When work of a really critical nature is undertaken, a lens of the best possible colour correction is called for, and by the use of fluorite for the internal positive lenses, combined with negative lenses of barium flint glass, it is possible to unite the red, green, and blue rays at the same focus, thus practically eliminating the secondary spectrum and producing an image free from colour—such lenses are called apochromatic. Whilst this type of lens can be so well corrected for chromatic aberration and also spherical aberration, there is a defect which unfortunately is inherent in the design, and that is the error known as the chromatic difference of magnification. When this aberration is present, a bright point in the outer part of the field will be rendered as a very short spectrum lying radially, because the magnification varies with the wave-length of the light. The magnitude of the effect is small near the centre of the field and is only noticeable in the outer parts. This aberration is due to the non-achromatized front lens or lenses of such an objective, and has to be corrected by the use of a compensating eyepiece (described later).

The reader would do well to supplement this brief treatment of the subject by reading the following article—Conrady, *Dict. Appl. Physics*, Vol. IV, pp. 202–236.

Measurement of Focal Length

The determination of the focal length of a microscope objective can be very conveniently carried out on the microscope itself, by employing a " magnification method ". The lens to be tested is screwed into the microscope, and an eyepiece micrometer is fitted into the draw-tube. (The

latter may consist of the ordinary Huygenian eyepiece the field lens of which is removed and a $\frac{1}{10}$ mm. glass scale placed in the focal plane of the eye-lens.) A stage micrometer is then put on the instrument and focussed, and the size of the image of a known interval of the object is measured up with the eyepiece—the magnification is noted down. The microscope draw-tube is then extended by say 40 or 50 mm. and the magnification again measured. If these two magnifications are denoted by m_1 and m_2, and the first and second positions of the draw-tube by v_1 and v_2 respectively, then the focal length f_o will be given by

$$f_o = \frac{v_2 - v_1}{m_2 - m_1}.$$

Tube Length

In order to obtain the best optical performance with a given objective it is very necessary to use it at its correct optical tube length; whilst this information is usually supplied by the makers, it frequently happens that, by the use of an incorrect thickness of cover-glass for instance, adjustment of the tube length may become necessary in order to correct the spherical aberration so introduced; and moreover, a practical test on the theoretical value given is always advantageous. The test is fully discussed in a paper*, the reference to which is given below. It consists in the examination of the extra-focal images of a " star " object, the latter being artificially produced by the minute holes present in a film of silver chemically deposited on the underside of a cover-glass.

The silver film slide is placed on the stage, illuminated with a strong source of light, and focussed with the microscope; a suitably small and round hole in the film is then selected. Assuming that the draw-tube is set to the value stated by the makers, the objective must then be moved just inside and outside the best focus position by the fine

* Martin, *Trans. Opt. Soc.*, Vol. XXIII, No. 2, 1921–22.

adjustment and the appearances thus produced are observed. By referring to fig. 22 (*a*) (which is a grossly exaggerated diagrammatic illustration of the union of the rays in the image formed by the objective), it will be seen that if the rays from all parts of the objective coincided in one point (i.e. freedom from spherical aberration), the appearances of the circular patch of light on each side of the best focus position would be similar. If on the other hand, appearances are seen resembling those in fig. 22 (*b*) and (*c*), " under-correction " or " over-correction " will be indicated; by altering the tube length and repeating the procedure a variation in the appearances will occur (an increase of the tube length usually gives a tendency to " over-correction " while decrease gives " under-correction "), and by continuing in this way, a position will be found when the extra-focal images appear symmetrical on both sides of the focus—this will represent the best tube length.

Cover-glass Thickness

It may possibly happen when such a test as the foregoing is made with a lens free from aberration that when the draw-tube has been set to the figure given by the makers there is still a lack of similarity in the appearance of the images on each side of the focus. This generally indicates that the correct thickness of cover-glass, for which the objective was originally computed, has not been employed; such an occurrence introduces spherical aberration, and makes itself apparent when using the higher power dry lenses such as $\frac{1}{6}$ in. to $\frac{1}{8}$ in. When the cover-glass thickness is too great, the effect is manifested by " over-correction " in the image; hence we seek to counteract it by diminishing the tube length. The effect of too thin a cover-glass will require to be compensated by increasing the tube length. The lower powers are not so sensitive to change in cover-glass thickness, and as the immersion objectives employ a medium between the front lens and the cover-glass similar in refractive index to the latter, the case does not arise for this type of lens.

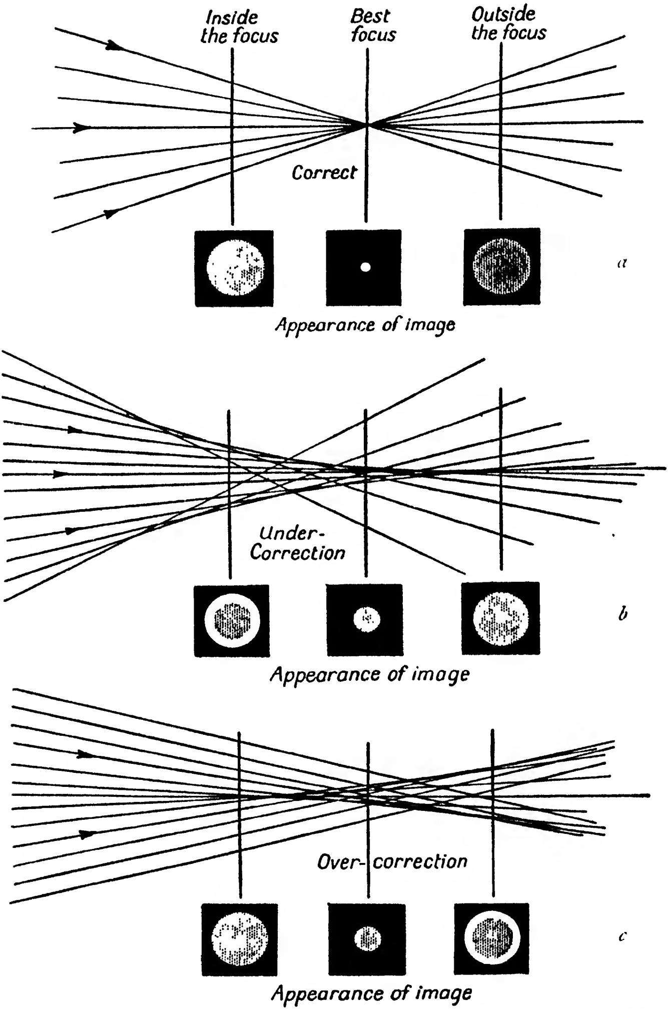
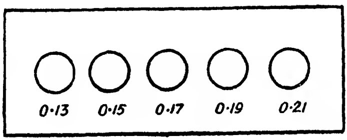


Fig. 22.—Diagrammatic Illustration (grossly exaggerated) of the Union of the Rays in the Image formed by the Objective, in the presence of zero, under-corrected, and over-corrected Spherical Aberration

Cover-glass thickness is, however, of importance, and it has been generally accepted that the usual thickness shall be 0·17 mm. Purchased cover-glasses vary from as low as 0·10 mm. to 0·25 mm. Therefore it is best to determine the correct tube length for a given objective by means of a special slide (fig. 23 (*a*)), on which there are cemented a number of silvered cover-glasses graded in thicknesses, and then to employ only the determined thickness for mounting desired objects thereafter. A quick test for cover-glass thickness is to "ring" them on a wooden table like a coin. Thick ones give a higher note. A useful piece of apparatus in this latter connexion is the modern type of Abbe test plate (fig. 23 (*b*)), which has a large range of thicknesses, provided by means of a glass wedge cemented to the slide. Alternatively the aberration so introduced may be compensated for by the *correction collar adjustment* fitted to some lenses. This consists of a device which provides the correction by a movement of the back component of the objective; the collar is usually engraved in terms of the cover-glass thickness. This device is not to be recommended for the most exacting work.

(a)



(b)

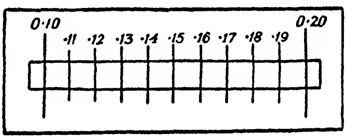


Fig. 23.—Slides for determining correct cover-glass thickness and tube-length

Eyepieces

The type of eyepiece most frequently used in microscopy is the Huygenian; it consists of two separate plano-convex lenses arranged as in fig. 24. The exact design of the lens depends on the glass used and the thicknesses, &c. According

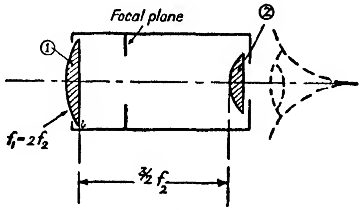


Fig. 24.—Huygenian Type Eyepiece

to elementary theory the separation should be half the sum of the focal lengths, but this condition is only approximately fulfilled in practice. The field lens has a focal length two or three times that of the eye-lens, and a diaphragm is situated in the focal plane of the latter; sometimes a glass scale (10 mm. divided into 100 parts) is also placed there.

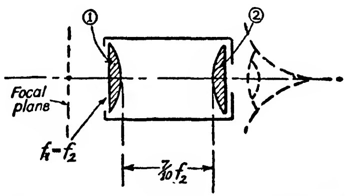


Fig. 25.—Ramsden Type Eyepiece

The Ramsden type (fig. 25) employs two plano-convex lenses of similar focal length, placed with their curved surfaces innermost, and separated by a distance equal to about seven-tenths of the focal length of either lens.

One of the chief differences between these two types of eyepieces is the fact that their focal planes lie within and outside the eyepieces respectively. The Ramsden type is

in consequence more generally used in eyepieces which are employed for measuring purposes, where, for instance,

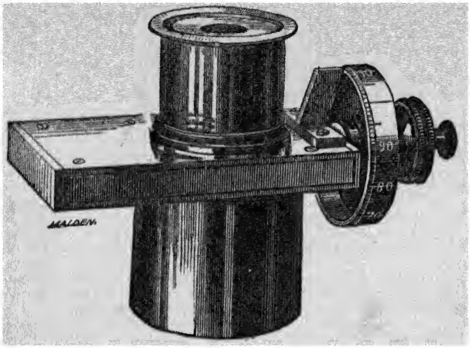


Fig. 26.—Micrometer Eyepiece

a cross web mounted in the focal plane is made to travel by a micrometer screw. Fig. 26 represents such a device known as a micrometer eyepiece.

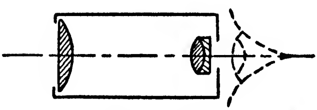


Fig. 27.—Achromatized Ramsden Eyepiece

The Ramsden type is frequently achromatized and then takes the form shown in fig. 27.

Compensating Eyepieces

Compensating eyepieces are so designed to be overcorrected for colour magnification, and are intended for use with apochromatic objectives in order to correct the chromatic difference of magnification introduced by the hemispherical shape of the front lens of such an objective. A practical way of distinguishing a *compensating* from an *ordinary* eyepiece (if not so marked) is to hold the ocular to the eye and observe an extended light source, when the former will show a red

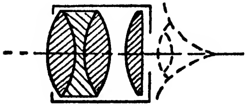


Fig. 28.—Compensating Eyepiece (Orthoscopic)

fringe to the edge of the diaphragm instead of the usual blue fringe seen with an *ordinary* type of eyepiece. The optical system of a compensating eyepiece is illustrated diagrammatically in fig. 28.

Projection Eyepieces

This is the name given to eyepieces specially intended for use in photomicrography, one of which is depicted in fig. 29. They are of the Huygenian type, but the eye-lens (generally achromatized) is fitted in a separate tube which has a spiral adjustment for focussing the diaphragm on the photographic plate and for giving a better state of correction of the final image. A graduated ring indicates the eye-lens movement according to the camera length employed. It is of importance to have a cap (fitting over the eye-lens) with a 1 mm. hole in it; this stop, which should lie in the plane of the exit pupil, is for removing stray light.

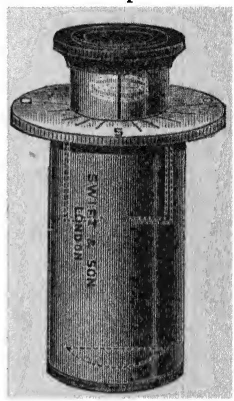


Fig. 29.—Projection Eyepiece

Other oculars suitable for this purpose and which are worthy of notice are the *Periplanatic* eyepiece (by Leitz) and the *Homal* (by Zeiss), both of which are intended to provide a flatter field on the photographic plate. The last named employs a "negative" lens system as the eyepiece, the curvature of field of which is of opposite sign to that produced in the primary image by the microscope objective. Thus the combined effect is to provide a distinctly flatter field than can possibly be obtained with a "positive" ocular. This eyepiece cannot be used for visual observation, but for photomicrographic work it is to be recommended; they are made in two powers, namely, focal lengths of —70 mm. and —20 mm.

Measurement of Magnifying Power

It has already been shown that the magnifying power of an eyepiece is given by the expression $M = \frac{D_v}{f}$ where D_v is 10 in. or 250 mm. and f the focal length of the lens system. It is therefore only necessary to obtain the equivalent focal length of the eyepiece in order to find its "power"; this may be done either by the method suggested on p. 4, or by using the focal collimator instrument also described. It is possible to use the microscope itself for such measurement* provided a special adaptor for carrying the eyepiece at the lower end of the body tube is provided. With an eyepiece so mounted, a glass scale on the stage, and by use of the draw-tube extension, the magnification method (as used for the focal length measurement of objectives, p. 20) may be employed.

Par-focal Adjustment

The eyepiece pushes into the draw-tube of the microscope until the knurled ring at the top of the eyepiece prevents any further movement. The focal plane of eyepieces of different powers will obviously vary with respect to this knurled ring, and consequently when an eyepiece is changed the focal plane of a new eyepiece will not necessarily coincide with the image plane of the objective; this necessitates refocussing the object, frequently by large amounts.

In order to avoid this, metal "sleeves" are fitted over the eyepiece tubes of the lower powers, so that they do not enter the draw-tube as far as the higher powers. The length of the metal sleeves so fitted is adjusted to bring the focal plane of each eyepiece coincident with the image plane of the objective. Such a set of eyepieces are then said to be in par-focal adjustment.

* Johnson, *Practical Optics for the Laboratory and Workshop*, p. 121.

CHAPTER IV

Numerical Aperture

It is now well known that the resolving power or structure-differentiating power of a microscope depends not so much upon the magnifying power obtained as upon the *numerical aperture* of the objective.

Numerical aperture (denoted N.A.) is an optical constant depending on the apical angle of the maximum cone of light which the lens can take up from a point of the object. It is defined as being equal to the product of the refractive index (n) of the medium outside the lens, and the sine of half the apical angle of the cone of light taken up by the objective. Thus (referring to fig. 30)

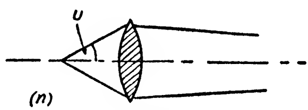


Fig. 30

$$\text{N.A.} = n \,.\, \sin \text{U}.$$

Methods of Measuring N.A.

The determination of this value is a relatively simple procedure, especially for the so-called "dry" objectives. In this latter case the quantity n becomes unity (on account of the outside medium being air), and it only remains to measure the angle U. This may be done in several ways, but one method which has proved satisfactory is illustrated in fig. 31.

A microscope tube M is mounted horizontally on an optical bench, and with the objective and eyepiece in the tube, the position of the "working point" O is found by focussing on a cross-line held in another optical bench fitting. A $\frac{1}{2}$-metre steel or wooden scale S is then set up at right angles to the axis of the optical bench and at a convenient distance, and two pieces of paper, P_1 and P_2, are hung on it.

A Ramsden eyepiece E is placed behind the microscope eyepiece and is adjusted until the "exit pupil" (which is an image of the back aperture of the objective) is seen sharply in focus. By moving the pieces of paper along the scale S, whilst observing through E, a position will be found when the edges of the paper just appear to pass out of the field of the objective aperture. If the auxiliary eyepiece E is not available, the eyepiece of the microscope may be removed in order that the appearance of P_1 and P_2 in the focal plane

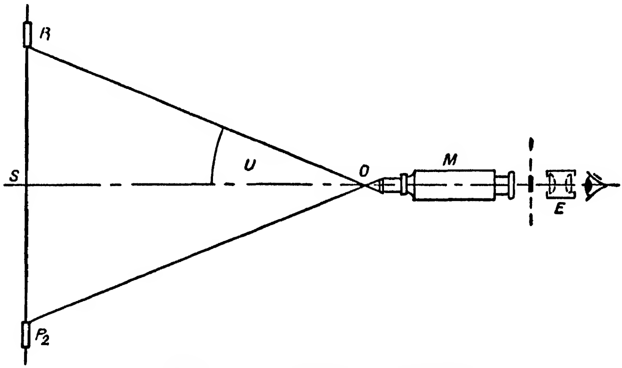


Fig. 31.—Method of measuring Numerical Aperture

at the back of the objective may be observed directly. When this has been done it is only necessary to measure up the distances P_1P_2 and OS and thus obtain the angle U from the fact that $\frac{P_1P_2}{2/OS} = \tan U$. The sine of this angle gives the numerical aperture of the lens.

There are, however, special pieces of apparatus made, known as apertometers, which are intended for use with the microscope alone; they rest on the stage, and are used in a similar way to that already described. These instruments usually are so calibrated that the numerical aperture may be read off directly. Two such devices are shown in figs. 32 and 33.

In Cheshire's apertometer—for use with dry lenses—the piece of white card as shown in the figure is clipped

Fig. 32.—Cheshire Apertometer

on to the stage and centred by focussing the zero mark with the microscope. The latter is then racked up through a

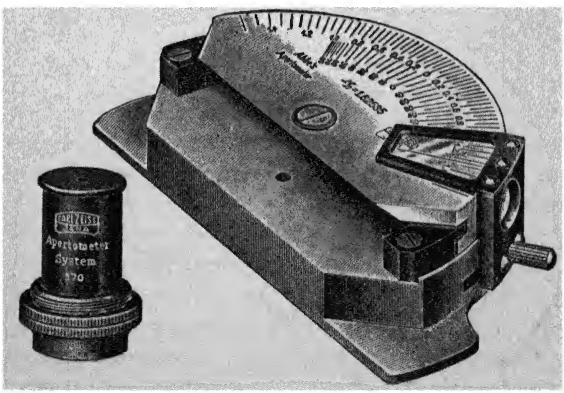


Fig. 33.—Abbe Apertometer

distance equal to 25 mm.—or alternatively a metal gauge, 25 mm. in length, is rested on the apertometer and the upper surface of the gauge focussed; this is the distance for which

the apertometer is calibrated. The eyepiece of the microscope is then removed, and the back of the objective observed with the eye positioned at the end of the tube; it is better to locate the eye by placing a piece of card (with a 2 mm. hole pierced in it) in the end of the tube. The appearance seen is that of the illuminated back lens of the objective, the diameter of which is crossed by black scale lines (incidentally it may be mentioned of apparent equal thicknesses). By reading off the divisions that appear just on the edge

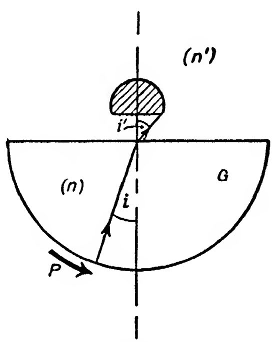


Fig. 34.—Principle of Abbe Apertometer

of the field the numerical aperture may thus be obtained. Another form of this apertometer is made for use with immersion lenses.

The Abbe apertometer can also be used both for immersion objectives and dry lenses. Its principle will be understood from fig. 34. A sliding pointer P is made to move round the edge of a semicircular glass block G, the microscope being focussed on the flat surface of the block. By observing the back of the objective (by means of an auxiliary lens screwed in the lower end of the draw-tube) the pointer is adjusted until it appears just on the edge of the field; its position is then read off on a scale round the edge of the block and gives the numerical aperture directly—very frequently the angle taken

in by the objective is also given. The calibration of the N.A. scale is obtained from the law of refraction, $n \, . \sin i = n' \, . \sin i'$.

The following table gives a list of objectives and their usual corresponding approximate numerical aperture.

TABLE II

Focal Length (mms.).	Numerical Aperture.
75	0·10
50	0·12
38	0·15
25	0·20
16	0·25
8	0·50
6	0·70
4	0·75
3	0·90
2	1·20
1·7	1·40

NUMERICAL APERTURE AND RESOLVING POWER

The resolving power of a lens may be defined as the smallest distance between two objects that the lens will render as separate images.

Owing, however, to the wave nature of light, a lens cannot produce a point image of a point object. Instead it produces a bright spot of light surrounded by diffraction rings (fig. 35). This effect is known as the Airy* disc. It has been determined that the radius of this disc $h' = \dfrac{0{\cdot}61\,\lambda}{\sin U'}$, where λ is the wave-length of the light concerned, and U′ the angle that the marginal rays (leaving the lens) make with the axis (see fig. 37).

Fig. 35.—Airy Disc

* Airy, *Camb. Phil. Trans.*, 5 (1834), 238.

If an intensity curve* (diagrammatically illustrated in fig. 36 (*a*)) showing the relative illumination of the central spot is drawn for the case when two point objects close together (fig. 36 (*b*)) are being observed by the lens, it can be shown

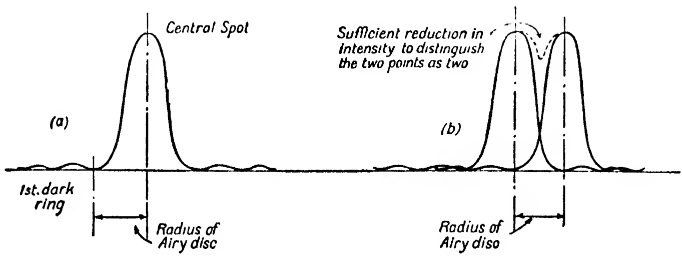


Fig. 36

that the two images will be separated by a darker region only when their centres are at a distance at least approximately equal to the radius of the Airy disc.

If this physical condition be applied to the case of the microscope, it will be seen how the resolving power of an

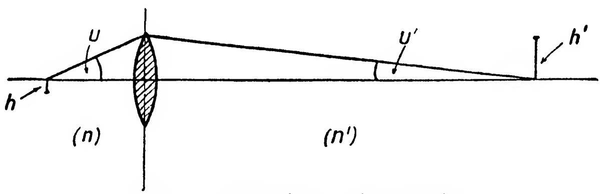


Fig. 37.—Illustrating "Sine Relation"

objective is deduced. Referring to fig. 37 the well-known "sine relation" will be

$$n \,.\, h \,.\, \sin \mathrm{U} = n' \,.\, h' \,.\, \sin \mathrm{U}',$$

where n and n' are the refractive indices of the media on the object and image sides of the lens respectively, h and h' the respective sizes of object and image, and U and U′ the

* Martin, *Trans. Opt. Soc.*, Vol. XXVII, No. 4, 1925–6.

corresponding angles that the marginal rays make with the axis.

In order, therefore, to find the smallest distance between two objects conditional with resolution, h' must be put equal to the radius of the Airy disc, namely $\frac{\cdot 61\,\lambda}{\sin U'}$.

Then

$$n \,.\, h \,.\, \sin U = n' \times \frac{0{\cdot}61\,\lambda}{\sin U'} \times \sin U',$$

but as the medium on the image side is air (of refractive index equal to unity)

$$h = \frac{0{\cdot}61\,\lambda}{n \,.\, \sin U},$$

and $n \,.\, \sin U$ is the numerical aperture of the lens so that

$$h = \frac{0{\cdot}61\,\lambda}{\text{N.A.}}.$$

Thus the resolving limit of a microscope objective is directly proportional to the wave-length of the light employed and inversely to the numerical aperture of the lens.

The physical constant 0·61 is a value which is subject to slight alteration, according to individual observation, in fact, in the light of modern work it is generally considered permissible to accept this value as 0·50. It should be noted that the above formulæ are derived on the assumption that the object points are self-luminous; this is not often the case in the microscope, and the theory of the resolution, especially of regular structures, may depend on other considerations. In particular, the resolving power may be considerably reduced by diminishing the angular aperture of the illuminating cone of rays from the condenser; the formulæ given are, however, a good guide provided the aperture of the objective is filled by light from the condenser.

That modern objectives do in practice come up remarkably near to their physical limit in resolving power has been shown by recent tests*, in which a method was employed for

* Johnson, *Journ. Roy. Micro. Soc.*, 1928, Vol. XLVIII, pp. 144–158

determining a numerical value for the limit of resolution. The following table shows the resolving power of objectives corresponding to various numerical apertures.

TABLE III

N.A. (visible light).	Separation of closest details just resolved (measured in microns).	Approx. number of lines per inch resolvable.
0·1	2·75	10,000
0·3	0·92	30,000
0·6	0·46	60,000
0·9	0·31	90,000
1·2	0·23	120,000
1·4	0·20	140,000
1·6	0·17	160,000

THE RELATION OF MAGNIFICATION TO RESOLVING POWER

Having determined what is the physical limit of resolution of an objective, it now remains to make this distance between two objects—or rather the angle subtended by them—visible to the eye.

It has been established—by Hooke, Helmholtz, and others—that the angle between two objects when they just still remain visible as two to the eye is one minute of arc. Therefore, two objects resolved by the microscope must *at least* subtend this angle to the eye, and the magnification must be sufficient to produce this.

Therefore,

$$M > \frac{1 \text{ min. (in circular measure)} \times D_v}{\left(\frac{0{\cdot}61\,\lambda}{\text{N.A.}}\right)}.$$

The above value is the absolute minimum necessary, but for *comfortable* vision it is desirable that the object shall subtend to the eye not 1 minute of arc, but something of the order of 4 minutes or even more. This means that the magnification should in general be approximately four times that indicated above. A value between these limits is usually known as *Useful Magnification.* There is no advantage to be gained by further increasing the magnification, as no further detail in the object can possibly be made visible to the eye.

Empty Magnification is the term given to such magnification which produces no useful result.

A good rule, in order to prevent too much empty magnification from being employed, is to multiply the numerical aperture of the objective by a thousand times, and not use a higher magnification than this (see Table IV).

TABLE IV

N.A.	Useful magnification limit (approx.).
0·1	100 ×
0·28	280 ×
0·50	500 ×
0·71	710 ×
1·0	1000 ×
1·3	1300 ×

The Use of N.A. as a Supplementary Method for Determining Magnification

The exit pupil of the microscope is observed as a bright disc just above the eyepiece when the objective is filled with light. If the diameter of the exit pupil is P, then

$$\text{Magnification of microscope} = \frac{2(\text{N.A.}) \times D_v}{P}.$$

This gives a means of obtaining the magnification if the numerical aperture of the objective and the diameter of the exit pupil are measured. The latter is obtained by a magnifier with scale (e.g. the scaleometer). In such measurements care must be taken to see that the whole aperture of the lens is illuminated, otherwise error will result.

Test for Efficiency of Magnification

It can be proved that if the diameter of the exit pupil is 2 mm. the angular separation of the "just resolved" image detail will be 1 min. of arc. If the angular subtense is increased, for comfortable vision, to 4 min., the exit pupil is diminished to 0·5 mm. If the diameter is less than this, unnecessary empty magnification is being employed; if greater, the eye may be more or less strained to see the resolved detail.

Depth of Focus

The depth of focus of an objective is of considerable importance in practice, for not only does it provide information as to the thickness of a given object which will be

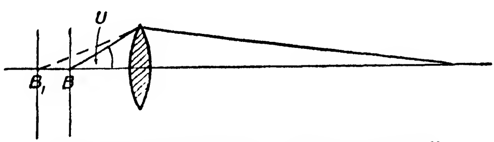


Fig. 38.—Illustrating "Depth of Focus"

in focus at one time, but it also decides the necessary delicacy of the fine adjustment of the microscope body.

Referring to fig. 38 it will be assumed that the microscope is focussed on a given plane of the object B. If the object point is shifted to B_1 an alteration will take place in the lengths of the central and marginal optical paths by which light can travel from the object point to the image plane.

According to Rayleigh, if the lengths of these paths differ

by more than a quarter of wave-length ($\lambda/4$), deterioration begins to be evident in the image. This criterion is possibly rather too severe, but it serves to set a limit.

Calculated on this basis, it can be shown that the total depth of focus *df* is given by

$$df = \frac{\text{Allowed difference of path}}{n \, . \, \sin^2 U/2},$$

where U is the angle made by the marginal rays with the axis in the object space, and n the refractive index of the medium on the object side of the objective.

Taking the allowed difference of path equal to $\lambda/4$ and the wave-length of the light, 0·00050 mm., the following Table V will give an idea of the depth of fairly sharp focus for a number of objectives.

TABLE V

N.A.	Depth of focus in air (millimetres).	Depth of focus in medium $n = 1{\cdot}5$ (millimetres).
0·25	0·0079	0·0122
0·50	0·0019	0·0030
0·75	0·0008	0·0013
1·00	—	0·0007
1·25	—	0·0004

CHAPTER V

Methods of Illumination of the Object

The microscope is fitted with apparatus for the illumination of the object in the form of (*a*) the mirror—plane on one side, and concave on the other—and (*b*) the condenser. The source of light itself is usually left to the choice of the microscopist, but the following are among those generally employed:

Daylight—cloud in north sky. (Conditions not similar at different periods.)
Paraffin oil lamp.
Incandescent gas mantle.
Opal-bulb electric lamp—for general use.
" Pointolite " electric lamp } for dark-ground work and
Arc lamp } photomicrography.

Probably the most convenient of these for general use is the modern opal-bulb electric lamp, as this gives an extended source of light of more or less uniform brightness. Such a lamp should be held in a suitable housing (fig. 39) to which an iris diaphragm is mounted, so that the size of the source may be controlled. Provision should also be made for carrying colour filters in front of the aperture, also some means whereby the intensity of the source may be adjusted. In this latter connexion a number of pieces of photographic negative of varying densities (cut to fit the slot indicated) will be found effective.

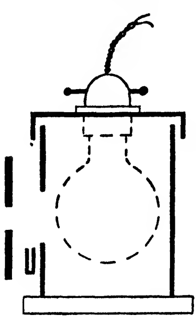


Fig. 39.—Microscope Lamp Housing

The following three conditions are of importance in the illumination of the object. Firstly, the angular aperture

of the beam from the condenser should be sufficient to illuminate the whole angular aperture of the objective, if required; secondly, only that portion of the object which is to be observed should be illuminated; and thirdly, in this region the illumination should be uniform.

Concave Mirror

The concave side of the mirror is only used for illuminating the object when the latter is to be observed with very low-power objectives, in which case the cone of rays required to fill such objectives can be supplied by the mirror. The arrangement of this type of illumination is shown in fig. 40; the distance of the source S is so arranged that the image of a pencil held in front of it, appears simultaneously in focus with the object on the stage of the microscope. It is usual with such illumination to have a series of stops (or alternatively a "cylinder diaphragm") placed as near to the object slide as possible; this provides a means of limiting the field of view to all but the required size.

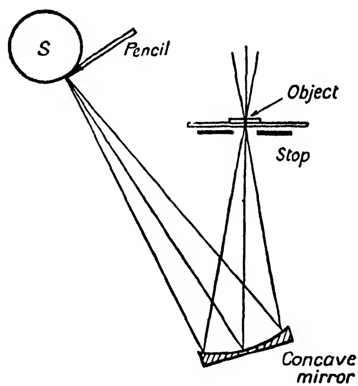


Fig. 40.—Illumination of Object with Concave Mirror

Substage Condenser

This consists of a system of lenses which collects the light from the illuminant and concentrates it on the object, providing a cone of rays of sufficiently wide an angle to wholly fill the objective with which it is being used. An image of the source is often formed (by means of the condenser) in the plane of the object if the source is sufficiently

large and of uniform brightness. The condenser is used with the plane side of the mirror, and is fitted with an iris diaphragm suitably mounted in front of the lens system. A very usual type is the Abbe condenser (fig. 41), which is useful for a great many purposes; it is not, however, corrected for chromatic or spherical aberration, and consequently an imperfect image of the source is formed in the plane of the object, resulting in much scattered light, which makes it somewhat difficult with a small source of light to secure a very even distribution of light into the objective. It has a numerical

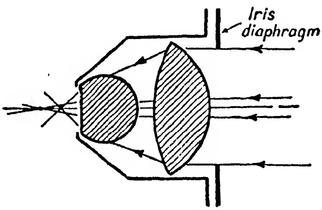


Fig. 41.—Abbe Condenser

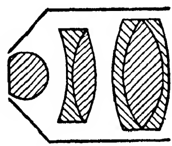


Fig. 42.—Achromatic Condenser

aperture of 1·0 and can thus be used with all dry objectives, but Abbe condensers can be employed up to 1·4 N.A. when used as immersion systems. For higher power and more critical work it is desirable to use an *achromatic condenser*. Such condensers are also corrected for spherical aberration, and on account of these corrections they become a somewhat complex system of lenses—one form is illustrated in fig. 42.

Frequently the upper lens or group of lenses can be unscrewed from the mount, and the remaining portion then constitutes a condenser of lower aperture and longer focus. Achromatic condensers are commonly given a numerical aperture of 1·0, but they can be made of still higher numerical aperture, and when " oiled " to the slide can be used with the highest-power immersion objectives.

Method of Illumination with Substage Condenser

When an extended source of light (such as an opal-bulb electric lamp) is used, the arrangement of the illumination is carried out as suggested in fig. 43. The object is first

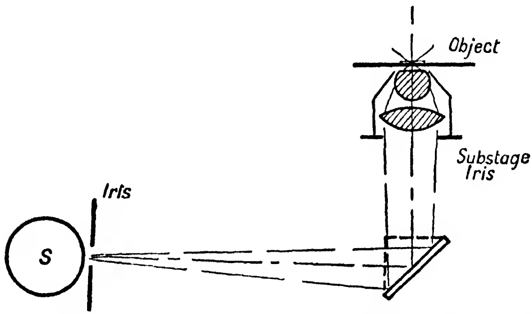


Fig. 43.—Method of Illuminating Object with Substage Condenser

focussed with the microscope, the substage condenser is then racked up or down until an image (of say a pencil) held in front of the lamp S is seen to come into focus. It is advisable to have an iris diaphragm mounted in front of

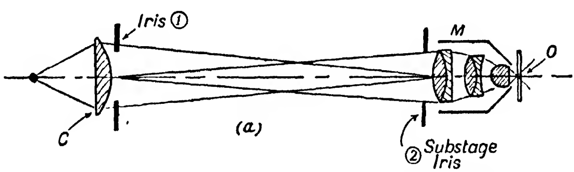


Fig. 44.—Illumination of Object when Source of Light is Small

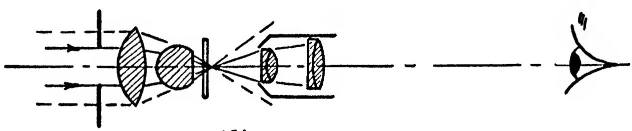
the lamp; this iris is focussed in the plane of the object and can then be closed down gradually until the desired field of view is obtained. Owing to multiple reflections produced by the mirror it is sometimes advantageous to substitute a right-angled prism for this (shown dotted in the figure).

When the source of illumination has only a small area—such as, for instance, a Pointolite lamp or Thorium burner

—it may be found that the whole area of the object is not filled with light, and consequently the size of the source is effectively increased by interposing a " Bull's-eye " or corrected condenser between the source and the microscope substage condenser (see fig. 44). The source and condenser C are so arranged that an enlarged image of the source is formed on and fills the back lens of the substage condenser M. In this way the lens C acts as the source of light and an image of it is formed in the plane of the object O by the condenser M; an iris diaphragm (1), placed against C, controls the size of the illuminated area of the object.

Use of the Substage Iris Diaphragm

The purpose of the iris which is mounted in the substage is to control the aperture of the condenser and thus to allow



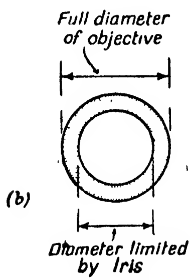


Fig. 45.—Illustrating the use of the Iris Diaphragm in the Substage

only sufficient light to pass through it in order just to fill the aperture of the objective. The admitting of a larger cone of rays than will fill the objective produces stray light and glare, and the image in consequence deteriorates.

Thus, in practice, when the object has been focussed, it is always necessary to remove the eyepiece temporarily and inspect the back of the objective; whilst doing this the iris is then expanded or contracted until the objective is just filled with light (fig. 45). Actually, however, for the sake of obtaining increased contrast in the image, the aperture

may be reduced to two-thirds that of the objective, but seldom less than this, otherwise bad diffraction effects will surround the image and a loss in resolving power will result. Very small condenser apertures are only to be used for special investigations, such as in seeking for evidence of very fine structures with oblique light.

Colour Filters

The purpose and use of colour filters in microscopy are as follows:

1. For producing greater contrast in the image, whether the object is stained or naturally coloured.

2. For producing monochromatic light. Objectives, although corrected for chromatic aberration, tend to give a better performance when used with light of one colour only; thus the use of a green filter is to be recommended where circumstances permit.

3. For increasing the resolving power by reducing the wave-length of the light employed, for instance by using a blue filter.

In connexion with purpose No. 1 referred to above, the following list of colour filters to use with objects of a particular colour will be found helpful.

Colour of object.	Filter to use.
Red	Green
Yellow	Blue
Green	Red
Blue	Yellow (sometimes red)
Brown	Blue
Purple	Green

Coloured solutions (contained in troughs which are variable in thickness) are also frequently used; for example, a saturated solution of acetate of copper gives a good blue-green filter.

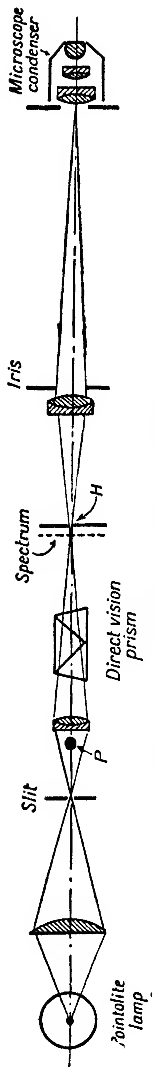


Fig. 46.—Illumination with Pure Spectral Colours

Circumstances sometimes arise when illumination is required by pure spectral colours, the wave-length of which can be changed at will; in such a case the apparatus is arranged as in fig. 46, from which it will be seen that to the right of the small round hole H, this is similar to the arrangement in fig. 44. The components to the left of H constitute a spectroscope, the spectrum of which is formed in the plane of the hole H; by rotating the spectroscope (as a whole) about some such point as P, any desired part of the spectrum may be brought over the hole H.

Illumination of Opaque Objects

When opaque objects are to be examined with the microscope, the substage condenser can no longer be used for illuminating the object, and other means have to be employed. Considering first the case of low-power objectives, where the working distance is relatively long, one method of illuminating the object is shown in fig. 47 (*a*), in which a beam of light is brought on to the object from an oblique angle. For a lens of slightly shorter working distance such illumination may be effected by a method depicted in fig. 47 (*b*) where a parabolic reflector is attached to the objective, and a parallel beam of light incident on the reflector is brought to a focus (free from spherical aberration) on the object. Whilst in the two foregoing cases the illumination is from one side only, annular illumination can be arranged as shown in

fig. 47 (*c*), here a paraboloid is fitted to the nose of the objective, and an incident parallel beam is received through the glass plate on which the object is mounted; this type of illuminator enables an objective of still shorter working distance to be employed. For illuminating an opaque object when viewing

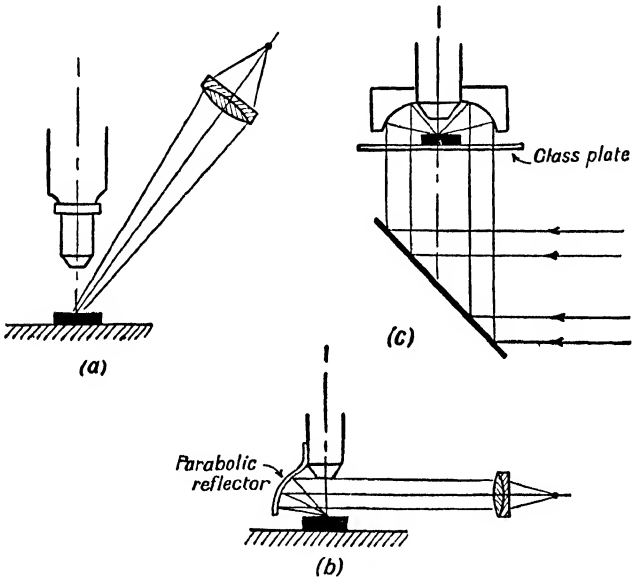


Fig. 47.—Illumination of Opaque Objects

this with an immersion objective it is necessary to employ other means, namely, the so-called "vertical illuminator", but this is dealt with in the section on the metallurgical microscope.

For the illumination of a relatively large area of an opaque object, the recent "top light * illuminator", by J. M. Preston, will be found useful.

* *Journ. Roy. Micro. Soc.*, Vol. LI, Pt. 2, pp. 115–118.

ROUTINE METHOD FOR CORRECT SETTING-UP OF THE MICROSCOPE FOR GENERAL USE

The setting up of the microscope in its proper manner is a matter of such importance that it was considered helpful to enumerate the various points to be attended to, and to arrange them in the order of carrying out the procedure.

1. Arrange the microscope and source of light (assumed in this case to be an opal-bulb electric lamp in housing) in convenient positions—distance of source about 12 in.

2. With all optical parts removed, tilt the plane mirror until the light is coming directly up the microscope tube.

3. Place the object on the stage, and observe same with a low-power objective (say a $\frac{2}{3}$-in. O.G.) and a $\times 10$ eyepiece, *taking care that the tube length is set to its predetermined best position* (if known) for the objective to be ultimately used.

4. Swing in the substage condenser and rack it up until the diaphragm in front of the source is seen in focus simultaneously with the object—a pencil moved about in front of the source may help this attainment.

N.B.—If an immersion objective is to be used later, it is as well to oil the slide to the condenser from the beginning—the method of doing this it to place a small drop of immersion oil on the top lens of the condenser and then to rack the latter slowly up until contact with the slide is made and the oil spreads out as a thin film.

5. Remove the eyepiece, and close the substage iris down as small as possible. Observing the back of the objective, the condenser can now be "centred" (by means of the screws provided) until the image of the hole in the diaphragm appears concentric with the aperture of the objective.

If necessary, replace the $\frac{2}{3}$ objective by the lens required for observation, focus, and remove the eyepiece again.

6. With the eyepiece still removed, gradually open out the substage iris, until approximately $\frac{2}{3}$ of the aperture of the objective is seen filled with light.

7. Replace the eyepiece, and observing the object, adjust the size of the iris situated in front of the source of light until all but the portion of the object immediately concerned with is screened from light. Remove the eyepiece once more and glance down the tube in order to see that this latter operation has not reduced the effective aperture of the objective.

8. Replace the eyepiece, and the instrument is now ready for critical observation of the object. A suitable light filter—depending on the colour of the object—may be inserted in the beam from the source, and one of the neutral tint glasses may be inserted as well if the illumination is still too bright for comfortable vision.

9. The same procedure, of course, applies for the higher-power objectives, and in this case also it is better to carry out the first five adjustments with a $\frac{2}{3}$-in. objective first. When using an immersion objective, a *small* drop of cedar-wood oil is placed either on the front lens of the objective or on the cover-glass, and the microscope very carefully brought down until "immersion contact" is made. Great care should be exercised in focussing or much damage can result. It is best for beginners to rack down the objective a little beyond the probable focus while watching from the side, then search for the image while moving the objective *upwards*. After use, the oil may be removed with cotton-wool soaked in xylol or (preferably) benzene and dried with a soft cloth (e.g. "selvyt").

Note on keeping Cedar Oil.—Immersion oil tends to thicken when kept for some time, when its refractive index tends to approximate more nearly to that of the cover-glasses; it should not be used when *very* sticky as the front lens may then tend to stick to the slide, and pull it up if racked backwards.

CHAPTER VI

Dark-ground Illumination

For microscopic vision, the importance of resolving power combined with the necessary magnification has already been mentioned; there is, however, a further point which has to be considered, and that is the *visibility* of the object under observation.

Visibility is dependent on *contrast between the object and its background*. This is already seen to some extent by the necessity of *staining* objects, by the use of colour screens, and by methods employed for reducing glare.

As an example of the inability to see an object due to lack of contrast, one may quote the familiar spider's web seen in the garden, which, if viewed against the sky as a background, is practically invisible; but if seen illuminated by sunlight against a dark background (such as a bush) becomes readily visible.

There are many objects similar to this (i.e. transparent and semi-transparent) such as micro-organisms and cell structures, which come into the sphere of microscopic work, and the general principle of observing objects brightly illuminated against a dark background has for some time now been adopted in connexion with microscopy.

It should be pointed out at once that the chief value in this method lies in the increase in visibility obtained—for instance, some transparent minute objects which were quite invisible by transmitted light, such as the filaments of *Coscinodiscus*, have been rendered visible by dark-ground illumination—but no actual increase in resolving power as regards the *separation* of detail is produced by dark-ground illumination. A serious drawback to this form of illumination is that it produces confusing diffraction effects round the image and thus makes the latter difficult to interpret, and

even at its best the method is apt to give deceptive images. Nevertheless, in spite of this fact, the rendering of the presence and approximate shape or form of the object causes considerable importance to be attached to the method.

In order to obtain dark-ground illumination with the microscope, the direct light which illuminates the object must not enter the objective—the only light entering the microscope must be that which has been scattered or reflected by the object itself. Thus it is necessary to make alterations in the usual form of apparatus employed for illuminating the object, namely, the substage condenser. For low-power lenses there is no great difficulty in this, and the dark field may conveniently be obtained by interposing an opaque stop in the centre of the ordinary condenser (see fig. 48). In this way the direct light is prevented from entering the objective, and the object appears illuminated against a dark background. Makers frequently supply a number of such stops of varying diameter which can be placed in front of the condenser in order to suit different power objectives up to about an 8 mm. Alternatively, a fitting known as the Traviss expanding stop (fig. 49) can be employed, which is a variable-sized opaque stop operated on similar lines to an iris diaphragm.

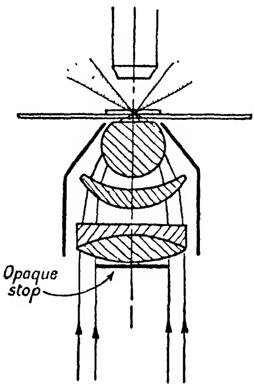


Fig. 48.—Dark-ground Illumination for Low-power Work

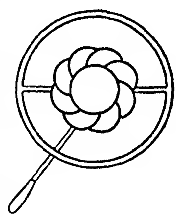


Fig. 49.—Traviss Expanding Stop.

For higher-power lenses such as the $\frac{1}{6}$ in. and immersion objectives, the foregoing device is not satisfactory, the reason being that the obliquity of the rays from the condenser is not great enough to be prevented from entering the objec-

tive, and also that owing to the sensitiveness of this type of illumination to colour effects, a *refracting* system (in which chromatic aberration cannot be entirely removed) as a dark-ground condenser is not satisfactory. The latter remark also applies in a less degree to low-power work. Hence, practically all dark-ground illuminators are of the *reflecting*

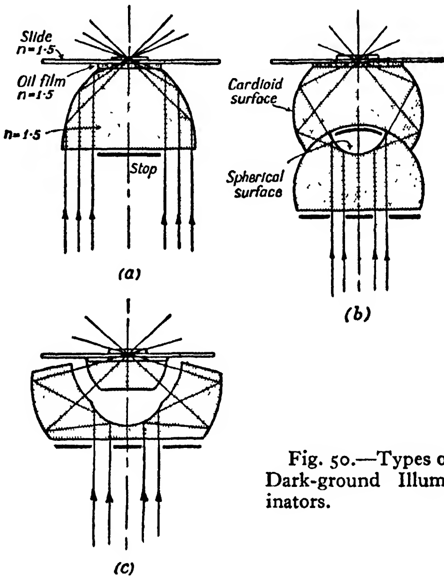


Fig. 50.—Types of Dark-ground Illuminators.

type. One of the simpler forms (depicted in fig. 50 (*a*)) is that known as the "Paraboloid", which, as its name implies, has a reflecting surface in the shape of a paraboloid of revolution, thus bringing incident parallel rays to a focus free from spherical and chromatic aberration. A stop blocks out the central rays. The slide is "oiled" to the surface of the "paraboloid" and must be of a definite thickness in order to bring the object into the correct plane where the rays unite. Figs. 50 (*b*) and (*c*) are other types, the former being known as the Cardioid (by Zeiss), which utilizes the principle that

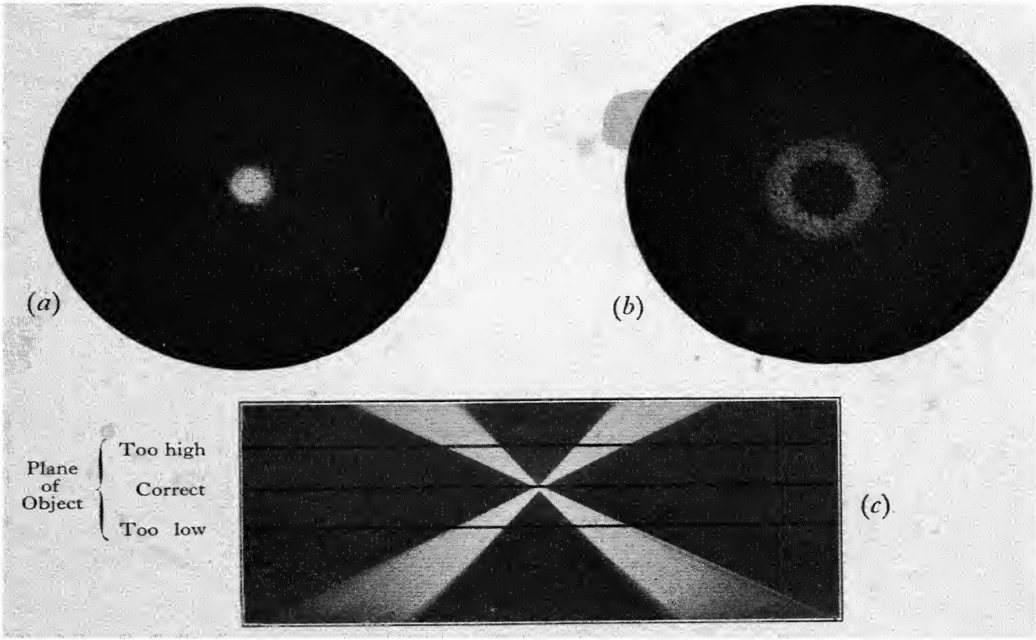


Fig. 51.—APPEARANCES SEEN WHEN ADJUSTING A DARK-GROUND ILLUMINATOR

Facing p. 52

the image formation produced by reflection from a spherical and cardioid surface is aplanatic, and the latter is the Beck focussing dark-ground illuminator. This last-named condenser has the advantage that a special thickness to the slide is not necessary, for by a movement of the lower reflector the concentration point of the rays can be raised or lowered through a range of something like a millimetre, and thus brought to coincide with the object. This fitting can therefore be used with various slides already made up.

Points to be Noted in setting up Dark-ground Illuminator

One of the main essentials in dark-ground work is the use of a bright source of light, and for this purpose there is no better illuminant (for convenience and steadiness) than the "Pointolite" lamp. A "bull's-eye" condenser is used in conjunction with this to form an enlarged image of the source on the aperture of the dark-ground condenser, the bull's-eye condenser acting as the effective source of light—as already described on p. 43.*

The object slide must in all cases be "oiled" to the upper surface of the illuminator, whether a low- or high-power objective is to be used for observation. Using first then (say) a $\frac{2}{3}$ in. objective for looking at the object, the dark-ground illuminator has to be *centred* and *focussed*. The appearance seen will probably be that of a ring of light (fig. 51 (*b*)) which will also very likely be displaced from the centre of the field; the latter can easily be rectified by adjustment of the substage screws. In the case of the cardioid condenser the centring is effected by an eccentric ring device.

The former appearance, which indicates that the union of the rays does not coincide with the plane of the object (see fig. 51 (*c*)), must be altered until that illustrated in fig. 51 (*a*) is attained. This is carried out by using the focussing

* In the case of the cardioid condenser it is better to have a roughly parallel beam falling on the aperture.

adjustment provided on the d.g. condenser; if the illuminator is of the non-focussing type sometimes this adjustment can be effected by the very careful raising or lowering of the substage mount, although on account of the small movement required this is not an easy procedure.

After these adjustments have been made the objective (which it is desired to use) is put on, and the instrument is then ready for use. With an immersion objective the settings made may require very slight readjustment, also it may be necessary to stop down a little the aperture of such an objective in order to prevent any direct rays from entering it. This can be done by means of a funnel stop which screws into the back of the lens mount.

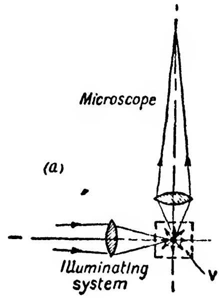
The Ultramicroscope

The term ultramicroscope is apt to be misleading, as it may convey the idea that with such an instrument one is able to see greater detail in an object than with an ordinary microscope. This, however, is not the case; rather is it the means of revealing the *presence* of very minute particles of matter, than any detail as to their structure. Whilst the smallest distance between two objects that can be seen is limited by the resolving power formula (given earlier), there is theoretically no limit to the smallness of an object which can be rendered visible provided sufficiently intense illumination can be obtained. The appearance representing a particle is merely that of a diffraction image or " Airy disc ". This instrument, which was really the forerunner of all present-day dark-ground illuminator methods, was introduced by Zsigmondy and Siedentopf* in their study of the behaviour of minute particles suspended in solutions in connexion with the subject of colloidal chemistry; also the study of the so-called Brownian movement by this means has confirmed the fundamental hypotheses of the kinetic

* Zsigmondy, *Zur Erkenntnis der Kolloide*, Jena, 1905.
Siedentopf, *Phys. Zeits.*, 1905, VI, 855; 1907, VIII, 85.

theory of liquids and gases, and contributed evidence of the existence of the molecule.

The principle of the instrument and the apparatus will be best understood by a diagram (fig. 52 (*a*) and (*b*)); the solution in which the suspended particles are present is contained in a suitable vessel V, in the side and top of which there are small optically worked windows. A very narrow and intense beam of light is allowed to enter the liquid cell through the side window and is brought to a focus in a plane coincident with the axis of the observing microscope; by this means the particles which lie in the illuminated area of the liquid



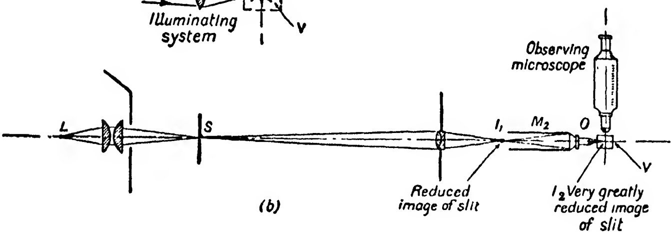


Fig. 52.—Principle of Slit Ultramicroscope

reflect and scatter some of the light, and their presence is rendered visible to the eye. The narrowness of the entering beam prevents large numbers of particles from being illuminated, and thus the amount of scattered light is not sufficient to spoil the dark-ground effect, which would result if a considerable area of the solution was illuminated. This condition is ensured by the arrangement of the apparatus. An intense source of light L is focussed on a horizontal slit S, and a reduced image of this is formed at I_1 by a suitable lens. This image is again reduced by another lens (namely, a micro-

scope objective), and a final image formed at I_2; the position of the latter can be adjusted by movement of the auxiliary microscope M_2, to which the objective O is attached, and thus the final image may be brought coincident with the axis of the observing microscope. It is now possible to employ a (water) immersion objective for use with the latter.

Measurement of Ultramicroscopic Particles

A recent extension of work on ultramicroscopic particles is that due to Gerhardt,* who has attempted to measure the size of mastic particles by an interference method, using a concentric dark-ground illuminator, when the particles would

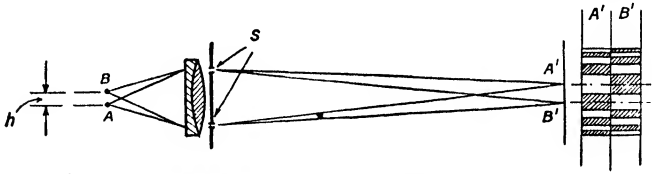


Fig. 53.—Interference Method for the Measurement of Ultramicroscopic Particles

really appear as small bright rings under sufficient magnification. His method was briefly as follows: if we think of a point object A (fig. 53) being observed by the microscope objective behind which are placed two slits S, interference fringes will be formed with the central fringe at A′; similarly fringes would be formed with the central one at (say) B′ by light originally emanating from B. By adjustment of the separation of the two slits it can be understood that the case will occur when a *bright* band of one set of fringes will correspond to the position of a *dark* band of the other set of fringes (indicated diagrammatically to the right of the figure), and a consequent disappearance of the interference bands will result. By co-relating the formula employed in Young's (interference) experiment with that of the sine relation for a lens system it can be shown that if A and B

* Gerhardt, *Zeitschr. f. Physik*, 35 (1926), 697, 44 (1927), 397.

represent the bright boundaries of the diameter of the object or particle (called here h), then for the lower limit for the disappearance of the fringes

$$h = \frac{\lambda}{4 . \mathrm{N.A.}},$$

where λ = wave-length of the light employed, and N.A. = numerical aperture of the objective.

Thus it will be seen that it should be possible to measure particles, the dimensions of which are only half the size of those which an objective would resolve in an ordinary way.

From the foregoing remarks on dark-ground illumination it will be seen that in spite of the fact that increased resolving power of the microscope cannot be hoped for by this means, yet it has proved and still is proving a helpful adjunct in many fields of research, on account of the contrast and consequent increased visibility obtained by its means.

CHAPTER VII

Photomicrography

The section under this heading deals, as its name implies, with the photography of specimens as seen in the microscope.

It has the great advantage of giving a permanent record of the object, although it lacks the " elasticity " of visual observation. We cannot, when looking at a photograph, change the focus slightly to observe the effect unless, indeed, a number of photographs at different foci are taken. Also, it is not easy to get photographic records of colour effects. On the other hand, a photograph can be examined at leisure, and in a detail which may not be possible with an object which is not of a permanent nature. In general, the micro-

scope has to be set up in a similar way and with the same care as for visual work, and then it is only necessary to mount a suitable housing—for carrying the photographic plate—behind the eyepiece, focus the image carefully, and expose the plate. The use of a " projection eyepiece " is, however, usually advisable.

A diagrammatic illustration of the image formation in photomicrography and the way in which the magnification may be derived in this case is given in fig. 54.

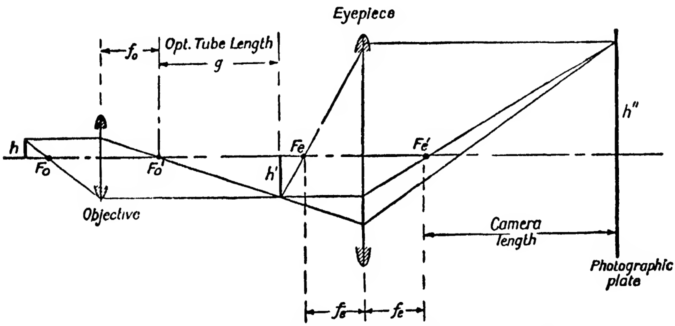


Fig. 54.—Formation of Image in Photomicrography

Magnification (for photomicrography)

$$= \frac{h''}{h} = \frac{h'}{h} \times \frac{h''}{h'} = \frac{\text{opt. tube length}}{f_o} \times \frac{\text{camera length}}{f_e}.$$

From the above it will be seen that, strictly speaking, the " camera length " should be measured from the focal point F_e' of the eyepiece to the plate, but in practice this point is very close to the eyelens, and therefore one can measure from the metal eye-cap without committing any serious error.

As an example of the use of the above formula, let us assume that there is to be used a $\frac{1}{6}$ in. objective (i.e. $f_o = 4$ mm.) working at a tube length of 160 mm. and with a $\times 10$ eyepiece (i.e. $f_e = 1$ in.), then the primary magnification

$m_1 = \frac{h'}{h} = \frac{160}{4} = 40$. And if a camera length of 10 in. is to be employed, then $m_2 = \frac{h''}{h'} = \frac{10}{1}$, thus giving a total magnification on the plate of 400 diameters. Similarly, if the camera length be made 30 in. in length, a final magnification of 1200 is obtained, and so on.

An alternative and *advisable* method of determining the magnification is to mount a stage micrometer on the microscope and photograph the scale divisions, and then to measure up on the plate the size of a known interval of the object.

Minimum Magnification in Photomicrography

The minimum permissible magnification necessary in this case is decided, not by the limitations of the eye (as deduced on p. 36), but by those of the grain of the photographic plate. It is, of course, well known that the size of grain varies for different types of plate, but it may be taken that the largest diameter of such grains is approximately 0·02 mm.; consequently, in order that the plate shall *safely* resolve the image, it is advisable to arrange the magnification so that the smallest distance between two points in the object rendered as two points by the objective, shall cover approximately nine or ten times the grain of the plate. Hence,

Minimum permissible magnification

$$= \frac{0{\cdot}02 \times 9}{\text{object interval resolvable by objective}}.$$

Thus, assuming the usual approximate numerical apertures,

for 1 in., necessary magnification $= \frac{0{\cdot}18}{0{\cdot}00125} = 144\times$.

" $\frac{2}{3}$ in., " " $= \frac{0{\cdot}18}{0{\cdot}00091} = 200\times$.

" $\frac{1}{6}$ in., " " $= \frac{0{\cdot}18}{0{\cdot}00036} = 500\times$.

" $\frac{1}{12}$ in., " " $= \frac{0{\cdot}18}{0{\cdot}00021} = 860\times$.

Arrangement of the Apparatus in Practice

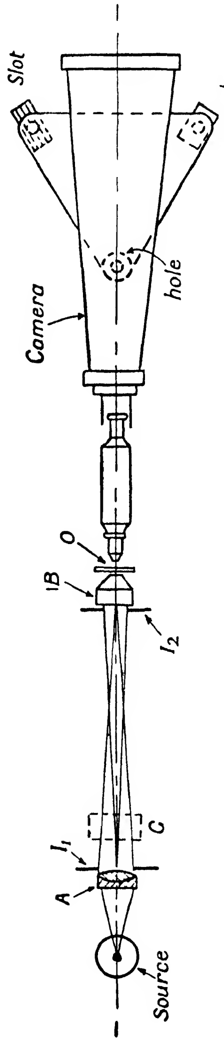


Fig. 55.—General Arrangement of Apparatus for Photomicrography

The general arrangement of the apparatus for photomicrographic work is illustrated in fig. 55. The illumination system on the left of the diagram is almost identical with that used for visual work, but it is generally necessary to have a fairly high-power source of light, such as a Pointolite lamp or one of the modern automatically-operating arc lamps, in order to obtain a reasonably short exposure. As before, an enlarged image of the source is formed on the back of the substage condenser B by a well-corrected lens A, the latter together with the iris diaphragm I_1 acting as the effective source; in turn, the condenser B forms an image of I_1 in the plane of the object O; thus, control of the illuminated area of the object is effected and glare prevented. I_2 is the ordinary iris in the substage, the purpose of which has already been explained. It may be advisable to introduce a cooling trough C containing water when the specimen may be liable to damage from heat. See also the principle of the arrangement shown in fig. 62, if the image of the source is not large enough to fill the aperture of the condenser.

The microscope itself as shown in the diagram is in a horizontal position, which raises the much discussed question as to the desirability of using it in this manner or in a vertical position for photographic work; the decision in this matter rests a great deal with the microscopist himself, nevertheless it should be pointed out that the horizontal position is advantageous in several respects. One of these is the fact that the illuminating accessories can be better aligned by this method, also the loss of light at the mirror is abolished; further, by having the base of the camera arranged with three steel balls which engage in a plane, slot, and hole fitting on the bench (see diagram), the camera can be entirely removed and replaced quickly at will, thus enabling easy access for the head when carrying out preliminary adjustments.

In connexion with the camera it may be pointed out that, if a camera length of (say) 26 in. be adopted, there is little need to have any adjustable bellows at all, for with such a length and by suitable choice of " eyepiecing " the necessary magnification for any of the more important objectives may be obtained. Such a length as suggested, however, avoids the use of high " eyepiecing" and thus agrees with the better practice of employing a fairly low-power eyepiece and extended camera rather than a high-power eyepiece and short camera length.

Table VI shows how such a scheme works out in practice when employing the normal types of photomicrographic projection eyepiece, namely, the nominal ×8, ×6, and ×4.

TABLE VI

Objective.	Necessary magnification for Photomicrography.	Primary magnification $\frac{\text{Opt. tube length}}{f_o}$	Focal length of eyepiece. f_e	Camera magnification $\frac{\text{Camera length}}{f_e}$	Magnification with eyepiece stated.
1″	144	6·4	30 mm. (× 8)	22	141
$\frac{2}{3}$″	200	10	30 mm. (× 8)	22	220
$\frac{1}{6}$″	500	40	40 mm. (× 6)	16·5	660
$\frac{1}{12}$″	860	80	60 mm. (× 4)	11	880

The mounting of the complete photomicrographic equipment is one which needs a little consideration; unsteadiness of the instrument is a rather more serious defect in photographic work with the microscope than is the case for visual observation, on account of the increased time required to record an impression of the object (i.e. the exposure of the plate). The apparatus should preferably be placed in a room situated in the basement of a building and as far from any machinery as it is possible to arrange; in spite of such precautions against vibration the passing of traffic nearby can also cause much inconvenience in this direction, and consequently it may be necessary in some instances to have the apparatus supported by spring suspension, or if it is only high-frequency vibrations which are troublesome this may be remedied by mounting the feet of the table on small platforms, underneath which are placed rubber (" Sorbo ") balls. It may be mentioned that it is advantageous to have the " photomicrographic room " capable of being made completely dark when desired.

Permissible Movement of the Photographic Plate without Change of Focus

It is possibly not generally realized that the exact locating of the photographic plate along the axis of the apparatus is not of great importance as far as precise focussing is concerned.

The reason of this is that the " focal range " or " depth of focus " *in the image space* in this case is a value of considerable magnitude (as distances in microscopy are reckoned).

The " focal range " in the image space of a lens system is given by the relation $\frac{\lambda}{n' . \sin^2 U'_m}$,*

where λ = wave-length of the light,

n' = the refractive index of the medium in the image space (in most cases unity),

* "Appl. Optics and Optical Design." *A. E. Conrady* (p. 141).

and U'_m = the angle that the marginal rays make with the axis (see fig. 56).

If this relation be applied to the case of the image projected on the photographic plate by the microscope (see fig. 56), it will be at once seen that the angle U'_m is a very small angle; for the diameter of the beam leaving the eyepiece would in a normal way be approximately 1 mm. or probably less,

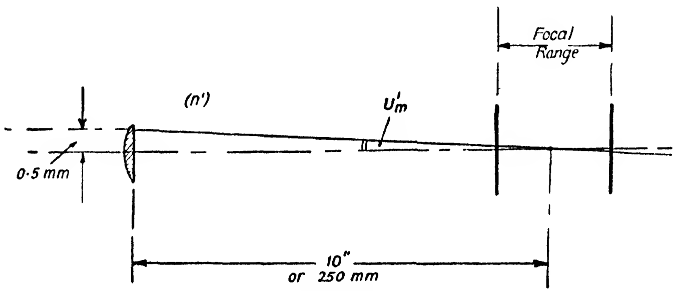


Fig. 56

and assuming (for this particular example) that the plate is at a distance of 10 in. from the eyepiece, then

$$\tan U'_m = \frac{0{\cdot}5}{250} = 0{\cdot}002, \text{ and } \therefore U'_m = 45 \text{ sec. of arc.}$$

So that,

the Focal Range for the plate

$$= \frac{\lambda}{n' . \sin^2 U'_m} = \frac{0{\cdot}0005}{1 \times (0{\cdot}002)^2} = \underline{125 \text{ mm.}},$$

i.e. <u>12·5 cm. or 5 in.</u>

Hence, there is an allowable movement of the plate of $2\frac{1}{2}$ in. on either side of the best focus position. For a camera length of 20 in., the value will be more than doubled. Thus it will be evident that there is considerable latitude in the movement of the plate without producing any serious change

in focus of the image, and as a result of this, any mechanical device for enabling removal and replacement of the camera as a whole need not be of a very accurate nature. Of course, the magnification will vary with any change of camera length.

Exposure

In photomicrographic work, the obtaining of a satisfactorily exposed negative is of primary importance; but whilst it is impossible to give a means of determining the correct exposure for the very numerous types of object and the conditions under which they may have to be photographed, it is possible to give some idea as to the way in which the exposure will change for given alterations in the optical system—such as, for example, change in magnification or numerical aperture.

In this connexion, therefore, the illumination per unit area of the image on the photographic plate is the point with which we are concerned. This, it can be shown, is approximately expressed as

$$\text{Illumination per unit area of image} \propto B \times \left(\frac{\text{N.A.}'}{m}\right)^2 \times t,$$

where B = the intrinsic brightness of the source,
N.A.′ = numerical aperture of the objective actually used, i.e. filled by condenser,
m = magnification,
t = transmission factor of the optical parts.

Thus, if it be imagined that the correct exposure for a certain object has been obtained empirically, then the new exposure required for any change in magnification or numerical aperture can be calculated from the above formula; for instance, by a doubling of the magnification (other things remaining the same) the illumination per unit area on the plate will be reduced to one quarter of what it was and consequently the exposure necessary will be four times that given previously.

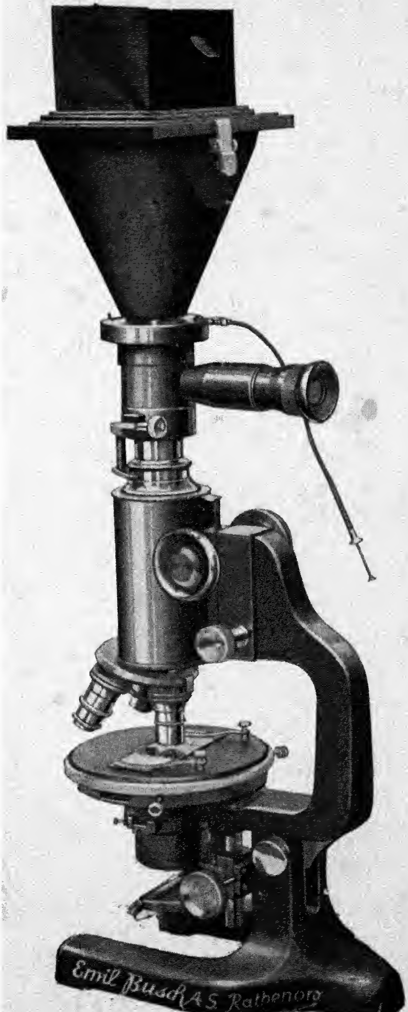


Fig. 57.—CAMERA ATTACHMENT FOR MICROSCOPE (By *Busch*)

Facing p. 64

Type of Plate and Development

The choice of the type of plate to employ presents some difficulty to those beginning such work, and here again this is dependent to some extent on the type of the object to be photographed. For the majority of cases, however, it is better to use a plate of relatively low "speed number" (say H. and D. 70)—for the sake of smallness of grain—and of the non-orthochromatic variety, as this makes for convenience in the dark-room where the permissible use of a red light facilitates manipulation. (Such plates frequently denoted as "ordinary" by any reliable maker will be found satisfactory.) For transparent or semi-transparent objects a *blue* colour filter, inserted in the beam illuminating the object, may be helpful both from the point of view of the optical performance of the microscope and of the structure-differentiation by the selective transmission or absorption of light of shorter wave-length. For coloured objects (whether natural or stained) the corresponding complementary colour filter should be employed and panchromatic plates used. In the development of the plate, if considerable contrast is the aim, hydroquinone developer will be found helpful, whilst pyrogallic acid or amidol developers should be used if better gradation is desired.

A useful camera attachment (now supplied by most makers) incorporating an observing eyepiece will be found a helpful accessory for photomicrographic work; an example (by Busch) is given in fig. 57. Such an attachment is easily fitted to the microscope, and it has the great additional asset of enabling observation of the object to be made right up until the instant of exposing the plate—in fact in the type illustrated observation is possible *during* the exposure, a point of considerable importance if the object has movement. A shutter and release are also contained in the camera unit.

CHAPTER VIII

The Metallurgical Microscope

The employment of the microscope in engineering has now become almost a necessity. The complexity of materials at present in use is so great, that without some such aid the accurate control of their manufacture and use would be impossible. The information which can be obtained by the use of the microscope in metallurgy is as follows:

(*a*) The examination of the structure of metals, and the alteration of that structure with service.

(*b*) Examination for defects (physical and mechanical) arising in manufacture.

(*c*) Control of the heat treatment which the substance is to undergo in manufacture (e.g. the "temper" of steels).

(*d*) Investigations of failures in use (e.g. the examination of a fracture).

Essentials of the Microscope for such Purposes

In most microscopic work, the light is transmitted through the object, but in the case of metals (the latter being opaque) the specimen has to be examined by light which is reflected from it, and in consequence of this the stand of the microscope for metallurgical work is modified. The substage condenser and mirror no longer become necessary, and the stage itself can be solid, that is, without an aperture through which the former may project. Fig. 58 shows a typical microscope for this kind of work, and will illustrate the foregoing remarks. In some instruments the stage is provided with rotational as well as the usual lateral adjustments, also it is capable of up and down movement (by means of a rack motion) for convenience in placing and replacing specimens of varying sizes. The rigidity of the

stage, in fact the instrument as a whole, is of considerable importance, as the placing on the stage of a heavy metal specimen may cause flexure of the mechanical parts and thus make focussing with a high-power objective almost impossible.

Methods of Illuminating the Object

The preparation of the specimen is dealt with elsewhere (p. 79), but suffice it to say here that the surface of the metal to be examined is polished and etched, and that such a surface is placed under the microscope and suitably illuminated. For observation with low-power objectives, that is, when the "working distance" is fairly large, it is possible to use means of illuminating the object as already described and depicted in fig. 47, (*a*), (*b*), and (*c*). More recently a device* (shown in fig. 59) has been introduced by Professor F. Hauser and made by Messrs. Busch, for the examination of specimens using very oblique illumination—in fact, dark-ground effects are yielded with this form of illuminator. Its principle will be understood from the figure. Incident parallel light passes through a glass plate on which the object is mounted, and strikes a flat mirror R of conical form; after reflection at this surface the light is again reflected at the parabolic mirror M and brought to a focus in the plane of the surface to be examined. In this way no directly reflected light enters the objective, but only that scattered by projecting portions of the specimen.

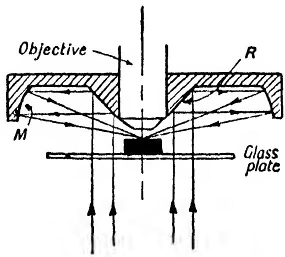


Fig. 59.—Oblique Illumination of Opaque Specimens

In any of these cases, however, the appearance of the image seen in the microscope may sometimes be of a mis-

* Hauser, *Tech. Instrument Bulletin*, Vol. I, Nos. 5 and 4.

leading nature, owing to shadows which may be cast by the minute irregularities in the surface when the illumination is oblique; moreover, when it is desired to use higher-power objectives and the "working distance" becomes correspondingly less, it becomes increasingly difficult—on account of the lack of space—to introduce the illumination by external means between the nose of the objective mount and the specimen. Therefore, a device known as a "vertical illuminator" is still almost generally employed for use in examining opaque objects; it consists in its simplest form of a plane

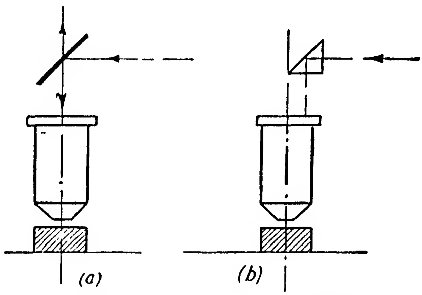


Fig. 60.—Vertical Illuminators

glass reflector (held in an adjustable mount) situated immediately behind the objective (fig. 60 (*a*)), and thus reflects the incident light down through the lens on to the specimen. The light returned by reflection at the latter again passes through the objective and through the "vertical illuminator" to form the final image. Sometimes the plane glass reflector is replaced by a right-angled prism, which is so arranged as to project over a part of the aperture of the lens (fig. 60 (*b*)); although by this means a more intense lighting of the specimen is obtained, there is a probability that the performance of the lens may be seriously affected by the covering of half its aperture with the prism. Therefore for all critical work the former type is to be recommended; it should be pointed out that both the glass and the surfaces of the reflector should

be of "optical quality"; but in this connexion, as it is so difficult in practice to make the surfaces of a small thin plate optically flat, it is suggested that more use be made of the collodion film as a "vertical illuminator". Such films if mounted on a framework slightly larger than the aperture required by the lens will serve admirably for this purpose; they are almost perfect from an optical point of view, and moreover are so thin (only a few wave-lengths of light) that no doubling of the image of the usual controlling iris is perceptible. They can be made by mixing equal quantities of methylated collodion and æther, pouring this on to an inclined flat glass plate and allowing to dry; the film thus produced is then floated off on to a water surface, the metal framework (already coated with shellac) placed down on to the film, and when the latter has adhered the whole is carefully lifted away from the surface of the water.

Much work has been done by Beck in connexion with vertical illuminators especially applied to metallurgical work, and particulars of this will be found in the reference* given below.

Arrangement of Optical Parts when using the Vertical Illuminator

Having dealt with the reflector for use in this work, other conditions connected with the ensuring of correct illumination of the object must be considered. The first of these is that the source of light or effective source should be situated at a distance from the objective equal to that of the primary image, that is to say, in fig. 61 (*a*) the distances marked A should be similar; in this way, an image of the source is formed in the plane of the object. Then, by having an iris diaphragm in front of the lamp as indicated, the illuminated area on the specimen can be controlled, so that all parts of the object other than that which the student desires to examine can be screened from light, thus preventing scattered light

* Beck, *Jour. Roy. Micro. Soc.*, 1930, Vol. L, pp. 319–322.

from entering the objective and "glaring" the image. This arrangement of the parts (fig. 61 (*a*)) would necessarily entail a considerable length of tube being attached at right angles to the microscope if the iris were to be carried as part of

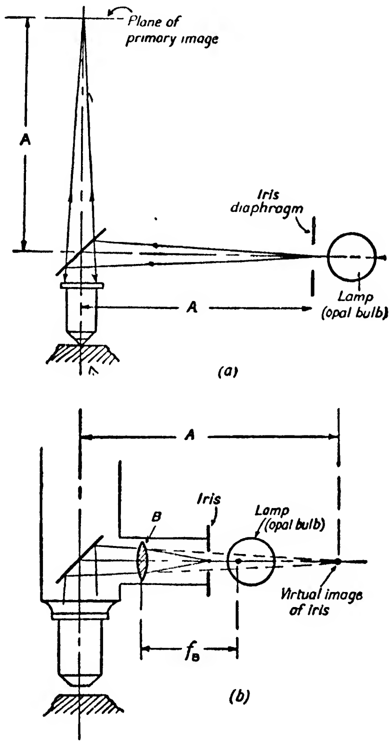


Fig. 61.—Illumination of the Object when using a Vertical Illuminator

the equipment, for the distance A in any ordinary microscope would be of the order of five to six inches. In this case, therefore, the iris and lamp generally form a separate unit and would have to be placed at the right distance from the axis of the microscope. As a matter of convenience, however,

the iris diaphragm and the reflector are frequently combined as one unit, which can be screwed into the microscope as illustrated in fig. 58. This can be accomplished by reducing the distance of the iris from the microscope axis by interposing a lens at B (fig. 61 (*b*)); the position of the latter is so arranged that a virtual image of the iris is formed at the long conjugate of the objective as shown diagrammatically in the figure, thus it is possible to use a short and compact piece of apparatus for the " vertical illuminator ".

When it is necessary to have more light available for

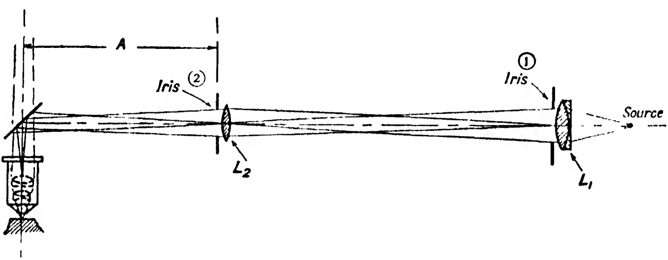


Fig. 62.—Arrangement of Apparatus for Metallographic Work

illuminating the specimen, such as, for instance, when photographic work is being done with a high-power objective, another system of the optical parts may have to be employed. The reason of this is that the more intense source of light which has to be employed is not in general of sufficiently large an area to fill the iris with light when placed close against it, as in the case of the opal bulb. The two sources now usually employed for this work are the Pointolite or the arc lamp, and the apparatus should be arranged as in fig. 62. An image of the iris diaphragm (2) is formed in the plane of the object as before; the source and condenser L_1 are arranged to give an enlarged image of the former on the lens L_2; which in turn forms an image of the iris (1) on the back of the microscope objective. By this means, both the aperture of the objective and the illuminated area

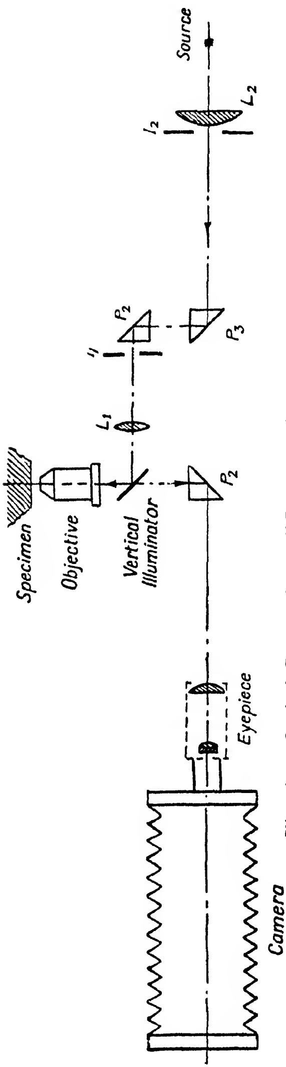


Fig. 64.—Optical System in an "Inverted Type" of Microscope

of the specimen may be controlled, this being done by alteration of the iris diaphragm (1) and (2) respectively.

The "Inverted" Microscope

Recent design in the metallurgical microscope tends towards the so-called "inverted" microscope, originally due to Le Châtelier. One of the chief points in the design of this instrument is the arrangement of the stage in an uppermost and easily accessible position, so that the metal specimen, irrespective of its size (within limits), can be conveniently brought into its correct position for examination (perpendicular to the axis of the microscope) with the minimum amount of trouble; in fact, some modern instruments (of similar principle) are so arranged that portions of the framework of some metal structure or device can be brought under observation *in situ*. A further point rendered possible by this design is the arrangement of both the illumination system and the camera in a horizontal position, and moreover to be in one and the

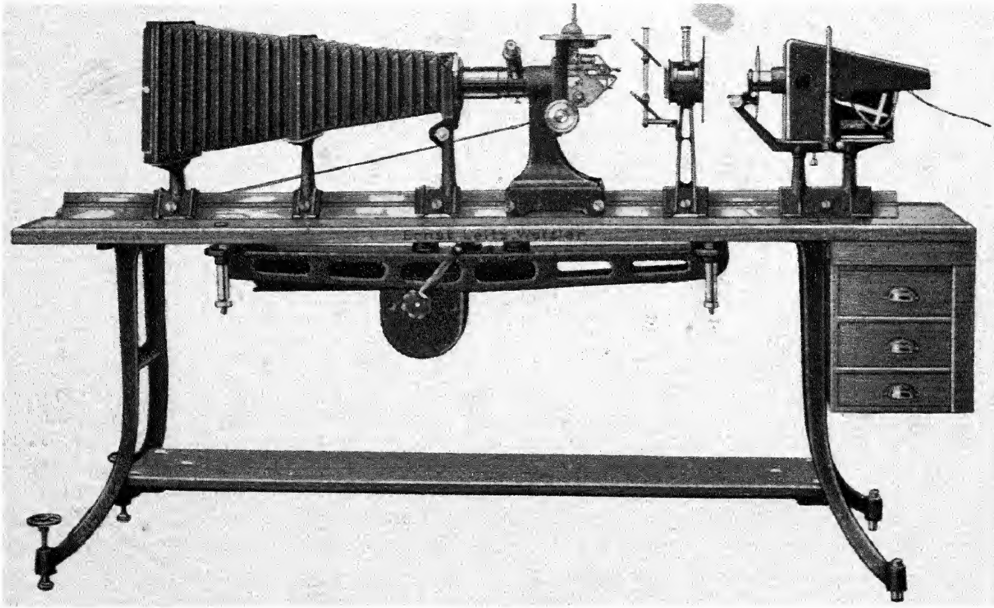


Fig. 63.—LARGE METALLOGRAPH (By *Leitz*)

Facing p. 72

same straight line; this enables the whole apparatus to be made of a more compact nature than has otherwise resulted in the past, and it is therefore very suitable for works use. Such instruments are now made by all present-day makers, and a typical example is shown in fig. 63, which is by Leitz; the optical system will in general be found similar to that illustrated diagrammatically in fig. 64.

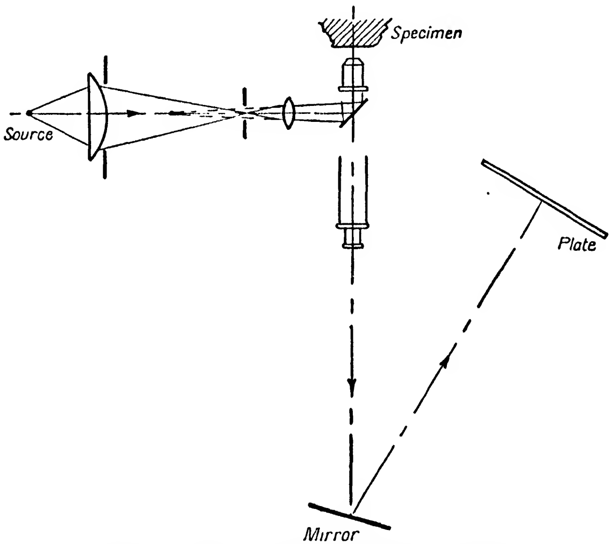


Fig. 65.—Vickers Metallograph (diagrammatic)

The objective is situated immediately below the specimen, followed by the vertical illuminator, beneath which is a right-angled prism; the latter deflects the beam into a horizontal direction, and the eyepiece and camera follow in the optical train seen on the left of the figure. On the right is the illuminating system, consisting of a lens L_1 and iris I_1, arranged as described in fig. 61 (*b*), two right-angled prisms P_2 and P_3, and a further lens and iris L_2 and I_2, and finally, the source of light itself. An image of the source

is formed by the condenser L_2 on the lens L_1, which produces an image of the iris diaphragm I_2 on the back aperture of the microscope objective; an image of I_1 (which acts as the effective source of light) is produced in the plane of the object by the combined effect of the objective and lens L_1.

It should be noted that a prism introduced into the convergent light between an objective and eyepiece may introduce undesirable aberration into the outer parts of the field, unless precautions are taken against this.

Another example of recent design in the inverted type of microscope is the Vickers metallograph, which is illustrated diagrammatically in fig. 65. A description of this instrument will be found in the reference* given below.

CHAPTER IX

Preparation of Specimens for the Microscope

Transparent Specimens

It is not intended here to give a lengthy account of the mounting of the many and varied types of object for the microscope, but rather a concise description of the more general methods now adopted for preparing a specimen for examination.

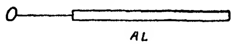
If we take the transparent or semi-transparent type first, it will be seen that there is an immense range of objects which come under this category; such as, for instance, Plant Life (embracing cell structure, woods, fibres), Insect and Animal Life, Diatoms, thin Rock Sections, Crystals of Salts, Bacteria, and so on, to quote only a few. Such objects

* *Journ. R. Micro. Soc.*, 1930, Vol. L, pp. 378–382.

resolve themselves into three classifications as regards mounting methods, namely, dry mounting, fluid mounting, and balsam mounting.

Before describing these methods, a brief list of the materials required will be given:

3 in. × 1 in. slides.
Circular cover-glasses (0·17 mm. thick)—cleaned and kept in alcohol.
Needle holder. 

Loop holder. 

Tweezers.
Canada balsam (diluted with xylol).
Xylol or benzol (in bottles).
Liquid cements: Gold size mixed with printer's black ink.
Shellac.
Bell's.
Turntable.
Spirit flame or Bunsen burner.
Stains in dropping bottles.
Camel-hair brushes (small).
Cotton-wool and boxwood sticks.

The primary consideration in all three of the methods is cleanliness, for it is surprising to find how easy it is to mount what is not required in the way of dirt and undesired substances.

Dry Mounting

It will be imagined for this operation that we have some object such as, for example, a portion of a leaf, leg of an insect, or a butterfly wing. Take a glass slide, thoroughly cleaned, place it on the turntable, and run a ring of gold size on to it—if the object is thick a metal or vulcanite ring should be affixed. Put a minute quantity of gum (gum tragacanth is best) in the centre of the ring and allow it to

dry. When ready to mount the object (which must be perfectly dry), breathe on the gum and place the specimen down on to the tacky gum, positioning it with a needle. Leave an hour or so for all the moisture to dry out, preferably by putting into a desiccator, and then attach the cover-glass; this is done by again mounting the slide on the turntable, putting a further ring of gold size or one of the above-mentioned cements on the previously made ring, and carefully placing the cover-glass down into position. When set, a final ring of cement may be put round the edge of the cover-glass, which process will both aid in sealing and also provide a finish to the slide. It is of the utmost importance that the specimen and cell be perfectly dry, if this is the case the object will remain permanent; but should moisture be left in the cell fungoid growths or cloudiness may result.

Balsam Mounting

For this type of mounting the object is embedded in Canada balsam, which is a pure form of turpentine. It is better to use this when it has been diluted a little with xylol; this thinning out enables bubbles to escape more readily, which fact is of considerable importance when mounting the specimen. If the object contains water, it must be entirely dehydrated (by successive immersions in alcohol), and then soaked in turpentine (to expel the air) before mounting; many types of object, of course, will not need this treatment. When the specimen is ready it is placed on the slide, a small quantity of balsam is carefully dropped on to it and the cover-glass placed on; the latter is gently pressed down with the aid of a small cork until the balsam has spread out as a thin film and oozed out from the edges. Careful observation (preferably with a hand magnifier) should now be made to see if any air bubbles are present in the balsam film; if there are, by the application of a little heat and pressure on the cover-glass with the cork, they can generally be removed; the superfluous balsam can then be wiped

off with a piece of cotton-wool dipped in xylol. By placing the slide in a warm place for twenty-four hours or so, the balsam will become hardened.

This type of mounting does not really require a varnished or cemented ring round the edge of the cover-glass, but this may be done, if desired, to afford more protection against displacement. Slides intended for use with immersion lenses should always be given a final ringing with shellac varnish, as this is the only cement which will satisfactorily resist the action of the immersion oil.

Fluid Mounting

This form of mounting generally applies to all those objects which are got up in a medium containing water, whether it be actually of a fluid or gelatinous nature. The method has the advantage that living specimens can be examined, or if dead, a more natural appearance is obtainable owing to the fact that the object is less likely to be damaged by pressure or removal of internal parts. There are various fluids which can be used, such as distilled water, dilute formalin, glycerin, solutions of neutral salts, and so on, but the choice is dependent on the needs of the case. For the larger type of object special cell-slides can be purchased for this work, and whilst no universal mountant can be given, either glycerin or a 3 per cent solution of formalin will be found to give excellent results in most cases. Some difficulty may be experienced in sealing down the cover-glass if the former be used, although this is soon overcome after a few trials; no such trouble will occur with the formalin solution, moreover this suits aquatic organisms better, and with it it is easier to get rid of air bubbles.

For the smaller type of object, such as the organisms dealt with in bacteriology, it is not generally necessary to have any special form of slide—although hollow-ground slides are frequently of use—as it suffices to use a plain slide or at most one which has been ringed with a very slight layer of cement.

The method of procedure for mounting bacteria or living cells may be carried out as follows. Put a drop of distilled water in the centre of the slide—sterilize "loop" (see p. 75) in flame—take minute quantity of culture from tube and spread out very thinly with the water already on slide—sterilize "loop" in flame—place cover-glass on and gently press down—examine with microscope to see if organisms are well spread out—if so, remove slide from stage, mop off superfluous liquid, and "ring" cover-glass with cement. If the organisms are to be photographed it may be necessary to put on the slide in the first place a solution of gelatine (3 per cent approx.) in order to keep them stationary.

Hanging-drop Preparation.—The observation of living bacteria (especially with regard to their mobility) is sometimes more easily carried out by the "hanging-drop" method. This consists in placing a drop of the culture fluid on to a clean cover-glass and then placing down over it one of the already mentioned hollow-ground slides. A ring of vaseline previously put on to the latter will ensure the adherence of the cover-glass to the slide; the whole may then be picked up and turned over ready for placing on the microscope stage.

The small and transparent nature of bacteria frequently makes them difficult to see in the microscope, and consequently *staining* has sometimes to be resorted to, in order to render them more visible. This process consists in putting a drop of solution (in which an aniline dye has been dissolved) on to the micro-organisms after they have been "fixed" on the slide—the latter operation being carried out by passing the slide once or twice through the flame—the stain is then poured off, the specimen washed with a few drops of distilled water and dried with alcohol. A small quantity of balsam is then applied, and a cover-glass placed on, and the object is ready for the microscope.

Of the several dyes which are employed for this purpose, methylene blue, fuchsin (red), and gentian violet are probably

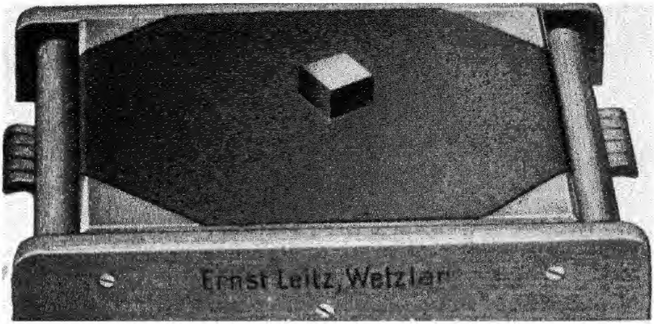


Fig. 66.—HAND POLISHING BOARD FOR THE PREPARATION OF METAL SPECIMENS

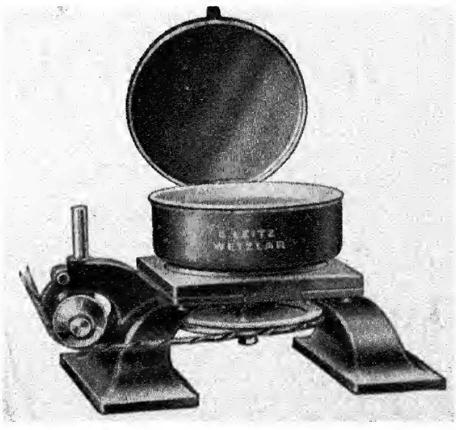


Fig. 67.—SMALL MACHINE POLISHER

the ones more generally used; they can be made up by dissolving 2 gm. of the dye in 100 c.c. of a 90 per cent solution of alcohol; or not infrequently these dye solutions are combined with carbolic acid, in which case a solution of the following proportions will serve as an example:

Methylene blue	1·5 gm.
Carbolic acid	5·0 gm.
Distilled water	100 c.c.
Absolute alcohol	10 c.c.

The reactions of the bacteria with the dyes often afford valuable evidence of the nature of the organisms.

Further particulars on this subject will be found in the following reference: *Bacteriology Technique*—J. W. H. Eyre.

Metal Specimens

The preparation of a metal specimen for examination with the microscope necessitates the careful polishing of its surface and then lightly "etching" it, in order to reveal the structure. The procedure for doing this is as follows:

A small piece (say $\frac{3}{8}$ in. long and $\frac{3}{8}$ in. diameter) of the desired metal is first sawn off, and one of its surfaces filed down as flat as possible, using a series of files of increasing fineness. The specimen is then rubbed vigorously on successive grades of abrasive paper (namely Herbert's "Blueback", Nos. 1, 0, 00) tightly stretched over a suitable baseboard (see fig. 66), the metal being continually rotated as the rubbing continues. When grade 00 has been reached the surface (on examination with a hand magnifier) should be reasonably free from scratches if the work has been done properly, and polishing may be commenced. This is most conveniently carried out by means of a small polishing machine (as supplied by most makers), one of which is depicted in fig. 67; a small table (to which is attached a closely woven cloth, chamois leather or selvyt) is made to revolve in a suitable trough. The specimen is held in contact

with the polisher, and a mixture of magnesium oxide and water is used as the polishing medium; by applying this at regular intervals during the operation the surface will gradually take up a good polish. Whilst a " cloth polisher " will serve most purposes, cases may arise when a pitch polisher will be necessary in order to produce a flatter surface than is attainable with the former method.

When a well-polished surface has been obtained, etching may then be carried out. This consists in immersing the specimen (usually for a few seconds) in a liquid that will gently attack the surface of the metal. It is then taken out of the etching bath, rinsed in alcohol, and dried off in a blast of air.

It would obviously be difficult to give a list of etching reagents for the many and varied metals and alloys, and therefore only a rough outline of those suitable for certain metals can be given in a book of this kind, such as provided by the examples given below.

For the steels.—4 per cent nitric acid in alcohol.

For the brass and copper alloys.—50 per cent ammonia and distilled water.

Aluminium and softer metals.—10 per cent caustic potash in water.

Fuller information on this subject will be found in the following references: Desch, *Metallography*; and Graeves & Wrighton, *Practical Microscopical Metallography*.

Mounting for Observation

When the surface has been prepared, the specimen has to be so mounted that it can be conveniently placed on the microscope stage and also that the surface to be examined is reasonably perpendicular to the axis of the microscope. One rather old but frequently adopted method is to place the specimen on a machined surface immediately over a recess bored in the latter (see fig. 68), and then to put on a metal ring (which has previously had its edges made

parallel). A piece of plasticine is carefully attached to the back of the specimen and arranged so as to project above the top edge of the ring, and the 3 × 1 slide then pressed down firmly on to the plasticine until contact is made with the top of the metal ring. The slide may now be lifted

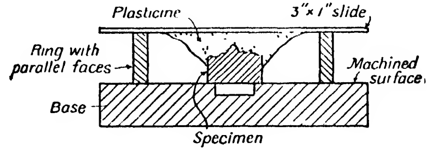


Fig. 68.—Method of Mounting a Metal Specimen for Examination

off with the specimen attached ready for placing on the microscope stage.

It will be evident, however, that the use of plasticine or some form of soft wax for holding the specimen is not altogether to be recommended on account of its liability to "give" or "creep", thus producing a change in height or tilt of the

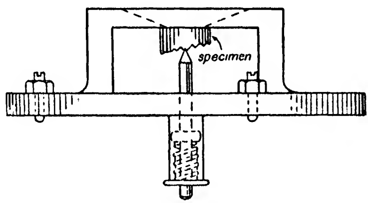


Fig. 69.—Alternative and Advisable Method of Mounting a Metal Specimen

surface whilst under observation; therefore it is desirable to employ a method on lines of that suggested in fig. 69, which will be found self-explanatory. The metal is held in a framework by means of the spring-plunger indicated; the upper cross-piece is well countersunk in order to allow the nose of the objective (e.g. $\frac{1}{12}$ in. oil) to approach the surface. If necessary, three screws may be fitted to the base in order to permit adjustment of the surface perpendicular

to the microscope axis. Such a mounting will securely hold the specimen and has proved satisfactory in practice.

The mounting of a small iron or steel object or a number of such objects together, e.g. wires, may be carried out by placing the objects on a glass plate which is rested on a magnet (see fig. 70); a mould is then placed round the specimens and molten Wood's metal is poured into it, the objects remaining in position owing to the action of the

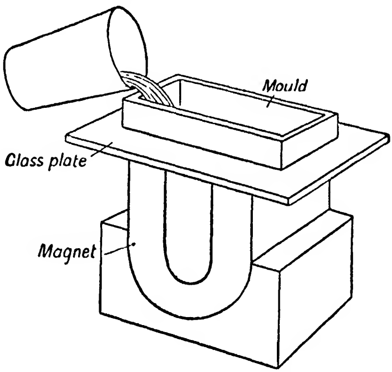


Fig. 70.—Method of Mounting a Number of Small Metal Specimens

magnet. When the Wood's metal has solidified and cooled down the mould may be removed and a block with the specimens embedded is obtained. The latter can then be readily ground and polished as already explained.

It is advisable to keep all metal specimens, when not in use, in a dry atmosphere to prevent corrosion; a desiccator will be found helpful for this purpose.

Blood-counting Apparatus

The blood test is of considerable importance in medical work, and is becoming increasingly employed for diagnosing many kinds of diseases, therefore the apparatus for counting the blood corpuscles will be briefly described. The hæma-

cytometer (as it is called) consists of a special slide made as indicated in plan and elevation views of fig. 71. A circular groove G is ground in the glass, and the remaining central island I is then very carefully reduced below the level of the slide, the amount being generally 0·1 mm.; by placing an optically worked cover-glass C on to the slide it will be seen that a thin cell $\frac{1}{10}$th of a millimetre in depth is thus obtained — this is the counting chamber in which the blood is placed. A small central portion of the surface I is very finely ruled in squares $\frac{1}{400}$th of a square millimetre each in area, in order to render possible the counting of the corpuscles. There are, however, various designs in the arrangement of the rulings, such as those by Thoma, Zappert, Breuer, and others.

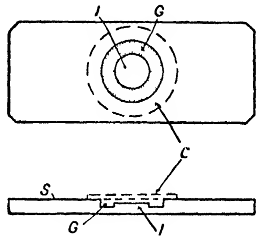


Fig. 71.—Blood-counting Slide

Two small pipettes (fig. 72) for providing the necessary

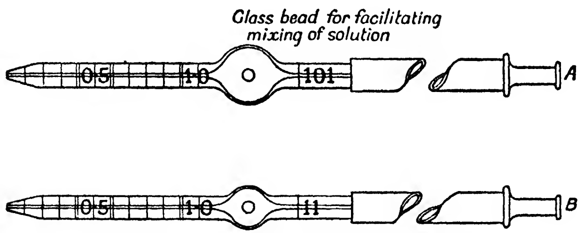


Fig. 72.—Pipettes used in Blood Dilution

dilutions of the blood are usually included in the equipment. When using the apparatus, the blood is first drawn up into one of the pipettes (A for *red* corpuscles counting, B for *white*) to the mark 0·5 or 1·0 depending on the concentration required. Then it is immersed in the diluting fluid (known as " Toisson's " solution) which is drawn up to the position marked 101 or 11 as the case may be—the dilutions generally

employed are 1–200 or 1–100 for counting red corpuscles and 1–10 for white. For filling the chamber, a minute drop of the mixture is very carefully blown out on to the centre of the counting chamber, and the cover-glass then placed on. By observation with the microscope it will now be found a fairly easy matter to count the corpuscles contained in each square of the rulings, although for the sake of accuracy it is usual to count say 100 squares.

By this means and by a simple calculation it is possible to obtain the number of corpuscles in 1 cubic mm. of undiluted blood; for from what has been said

Number of corpuscles in 1 cubic mm. of undiluted blood

$$= \frac{\text{rate of dilution} \times \text{number of corpuscles counted}}{\text{number of squares counted} \times \text{volume of each square (namely } \frac{1}{4000} \text{ cu. mm.)}}$$

An alternative method to the dividing of the counting chamber into small squares is to employ a squared graticule placed in the focal plane of the microscope eyepiece, and to arrange the primary magnification such that a definite number of squares correspond to 0·1 mm. square in the object (this being done by the aid of a stage micrometer and adjustment of the tube length). If the depth of the cell in which the blood solution is placed is 0·1 mm., a convenient volume (namely a cubic tenth of a millimetre) of fluid for counting purposes is thus obtained. Such graticules, of a chess-board pattern with tinted alternate squares, suggested and made by Mr. Rheinberg, have greatly assisted the work by this method.

More recently, a "differential blood plate" has been introduced by Messrs. Busch, which consists of a number of very fine parallel rulings instead of squares; a certain amount of capillarity is produced by these engraved lines, and in consequence, the corpuscles are sucked down and lie clearly and distinctly between the lines. The corpuscles thus ranked are counted by searching one line after another by means of the mechanical stage, and it is claimed that in this way mistakes are less liable to be made.

CHAPTER X

Binocular Microscopes

The general idea of adapting the microscope for binocular vision may be said to date back to the time when Wheatstone introduced his epoch-making ideas on stereoscopic vision some eighty years ago. The instrument was then primarily intended for producing stereoscopic vision, and for some time took its form in two separate microscopes arranged closely together (and slightly convergent) for viewing the same microscopic object with both eyes. It is only in comparatively recent times that considerable strides have been made in the development of the binocular microscope, and from the early low-power instrument, a type has emerged which enables work of the highest powers now to be carried out, thus giving the worker the choice of using for all kinds of examinations both eyes if he so wishes.

Modern instruments may be classified under two headings, namely, those which are intended to give stereoscopic vision (that is, the rendering of solidity, depth, and more natural appearance to an object), and those which present to the eyes two identical or congruent images but without the stereoscopic effect being present (i.e. they should be definitely non-parallactic).

Descriptions of some of these instruments are given in the ensuing section.

Binocular microscopes may be divided into three types, namely, those which have two body tubes and two separate objectives, those with two body tubes and only one objective, and those with but a single body tube and a binocular eyepiece fitting.

As an example of the first of these the Greenough type may be quoted (fig. 73). The instrument is generally intended for low-power work, and as in this case the working distance

is quite large, it is possible to arrange the objectives side by side. The body tubes are fitted with the Porro erecting system of prisms in order to make the instrument more useful in dissecting work and to produce a stereoscopic rather than a pseudoscopic effect; the combined effect of an erect image and stereoscopic vision aids very considerably work of this nature. The use of the prism system enables a convenient adjustment to be made for inter-ocular distance.

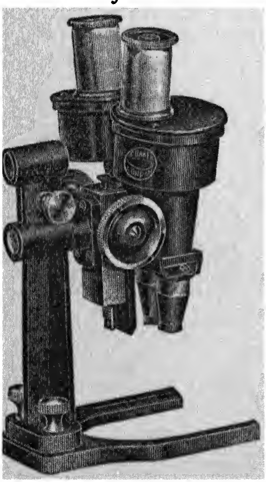


Fig. 73.—Greenough Binocular Microscope

There were various stages of development in the attempt to make binocular vision possible when using higher-power objectives, such as the method suggested by Wenham of interposing a prism behind the lens (see fig. 74), and that of Powell and Lealand depicted in fig. 75. In the former case, owing to the inability of placing the prism close enough to the back lens of the objective, its use was precluded for anything much above a $\frac{2}{3}$ in., nevertheless this was an advance on the power obtainable with the two-objective method. Stereoscopic effects could be produced, but only at the expense of loss of aperture.

The undesirability of using only half the aperture of the lens for producing either of the images was surmounted by the scheme of Powell & Lealand (fig. 75). The "stereoscopic" effect disappears in all cases where the image presented to each eye is formed by the full aperture of the one objective. It can only be restored by the use of special stops behind the eyepieces. These are sometimes supplied by the makers (see below). In Powell & Lealand's instrument, prism A (large enough to receive light from the whole lens aperture) was arranged so as to divide the beam into two by refraction

and reflection, the latter part being deviated to one of the oculars by means of the second prism B. Whilst this device was certainly a step in the right direction, it suffered from a rather troublesome defect due to the fact that only a small percentage of the light incident on the first face of the prism A is reflected and consequently the intensity of the light received in one eye is much lower than that of the other. This difficulty was overcome by the arrangement employed

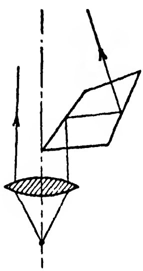


Fig. 74.—Wenham's Prism for Binocular Microscopes.

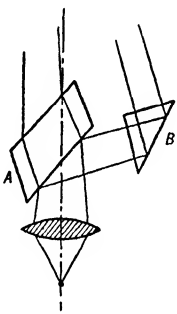


Fig. 75.—Powell and Lealand's Prism Device.

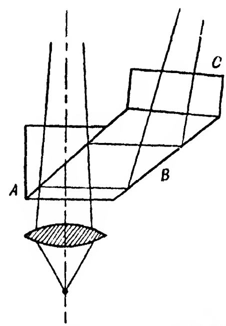


Fig. 76.—Arrangement of Prisms by Beck.

by Beck (see fig. 76), in which the intensity of the beams was corrected by "half-silvering" the surface (indicated in the diagram) between the two prisms A and B cemented together. The relatively lower position of the image plane of the right-hand beam is counteracted by interposing a parallel plate C (attached to B), the thickness of which is so arranged as to bring the focal planes at their correct distances from the objective to enable similar magnifications to be produced and that eyepieces of similar type and power may be employed. A further point of interest about this instrument is that the prism system just described can be conveniently displaced at will and the microscope then used as a monocular instrument. It will be seen

that this form of binocular microscope enables all powers of objective to be used, and is an example of the second of the types already spoken of; it is illustrated in fig. 77.

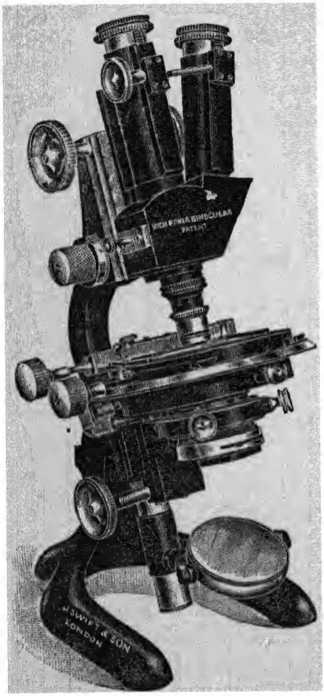


Fig. 77.—High-power Binocular Microscope

The third class deals with the adaptation of the binocular eyepiece to the existing monocular microscope. The first of these to be considered will be the Abbe type, illustrated in fig. 78. The beam from the objective is split up into two by means of the combined prism ACBD. The air film (which should be less than 0·01 mm.) produces partial reflection of the beam to the right, and at the same time allows of partial transmission. In order to compensate for the variation in position of the two images *e* and *f* (due to the lateral displacement XY) it is necessary to employ two different types of eyepiece, namely, an Huygenian and Ramsden respectively as depicted in the figure. These should be of similar power or focal length, so that equal magnification is maintained for each eye. Interocular adjustment is provided by action of the knurled head H.

For arduous examinations—especially when using high magnifications—it is desirable not to impose all the work on one eye alone, and therefore the use of both eyes is to be recommended for such operations. One means for fulfilling this requirement consists of a binocular "head"

which fits into the ordinary monocular body, its outward characteristic being that the two eyepiece tubes are parallel and not convergent. The internal system (although varying slightly for different makers) may be represented as shown in fig. 79, in which the beam received from the objective is separated into two at the semi-silvered interface AB of the "Swan cube", and the two beams brought to foci at similar heights, thus enabling the same types of eyepieces to be used. An advantage claimed by the use of the parallel

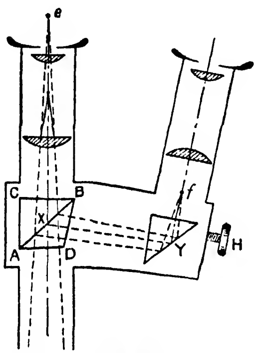


Fig. 78.—Abbe Stereoscopic Eyepiece

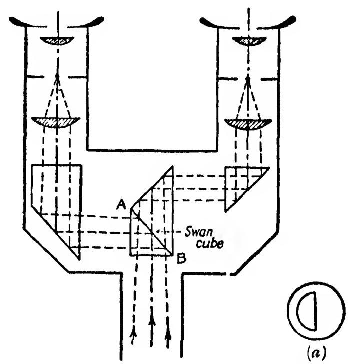


Fig. 79.—Binocular Eyepiece Fitting (diagrammatic)

arrangement of the eyepieces is that by its means no convergent action is induced on the observer's eyes, and in consequence, no accommodation effort is forced on them. Thus the muscles are allowed to remain in a state of complete rest, and therefore the eyes are able to observe free from any strain; so that, from an hygienic point of view, this attachment to the microscope would appear to be of considerable value when prolonged examinations are to be made.

It is possible, if desired, to produce stereoscopic vision with this device. The action is brought about by the fitting of semicircular caps (see fig. 79 (*a*)) in the plane of the "exit pupil" of the eyepieces. When the inner portions of each

beam are occulted by the opaque segments of the caps stereoscopic relief will result, and incidentally if the diaphragms are reversed, a pseudoscopic effect will be seen. These conditions were first made clear by Abbe in a paper* referred to below.

To supplement this brief treatment of the subject of the binocular microscope, the reader would do well to read a paper by F. Jentzsch given in the *Journal of the Royal Microscopical Society* for 1914, pp. 1–16.

In general, however, one must conclude that even with present-day refinements of this type of instrument, the one advantage of using two eyes does not seem to outweigh the loss of several advantages possessed by the monocular microscope. For critical work the optical definition of the image is necessarily never as good with the former as with the latter instrument; moreover, the additional cost of the binocular microscope or attachment makes it usual to purchase a monocular instrument. Nevertheless, the merit of using two eyes, whether for stereoscopic or non-parallactic vision, should for certain purposes always be borne in mind.

CHAPTER XI

Polarized Light and the Microscope

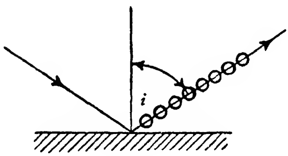
The application of polarized light for use in conjunction with the microscope has been the means of adding very considerably to the usefulness of the instrument for particular branches of work. Amongst these perhaps geology must have the first claim, for Sorby's original work in this connexion dates back to 1860, and much valuable work has been done with the polarization microscope since that time; nevertheless in recent years the microscope fitted with polar-

* Abbe, *Journ. R. Micr. Soc.*, 1881, pp. 203–11.

izing apparatus is also becoming of aid to the worker in biology and metallurgy.

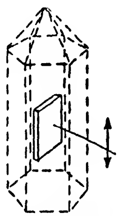
Before dealing with the instrument itself for this work, a few remarks must be made on polarization phenomena, but without, however, going into a lengthy treatment of the subject.

The production of plane-polarized light (i.e. radiation in which the vibration directions perpendicular to that of



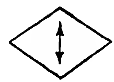
Reflector

Vibrations parallel to reflecting surface, i.e., perpendicular to plane of paper.



Tourmaline

Vibrations parallel to axis of symmetry of crystal.



Nicol Prism (end view)

Vibrations parallel to short diagonal.

Fig. 80.—Types of Polarizing Devices

propagation are confined to one plane only) may be carried out in several ways; probably the three chief methods are:

1. By reflection at a polished surface at the appropriate angle—($n = \tan i$, where i is the polarizing angle and n the refractive index of the material at which reflection takes place). For glass $i = 56\frac{1}{2}°$ approximately.

2. By transmission through a plate of tourmaline cut parallel to the optic axis.

3. By transmission through a Nicol prism or modification of this form of prism.

The vibration direction of the light after leaving such "polarizers" is illustrated in fig. 80. If any two of these polarizing devices are arranged in optical train and so orientated that their vibration directions are parallel, light will pass to an eye placed behind the second polarizer (or as

it is generally known, an "analyser"), but if one of them be rotated so that its plane of vibration is now at right angles to that of the other, no light will now pass to the eye. The latter condition is usually spoken of as "crossed Nicols", and the former "parallel Nicols".

It is the appearances which occur when certain kinds of objects are placed between the polarizers that provide the microscopist with considerable aid in the identification of a specimen, and this in a manner which it would not be possible to do by ordinary observation with non-polarized light. For instance, the identification of minute particles of crystals, minerals, &c., is effected by this means; and differentiation in biological specimens such as of fibrous membranes embedded in other tissue may be greatly facilitated.

A microscope for such work, then, consists of the ordinary type, but with the necessary polarizing devices fitted. In a simple form of the instrument merely two Nicol prisms may be employed, one mounted in the substage immediately in front of the condenser and the other conveniently held over the top of the eyepiece, but which can be swung out of position when desired. Both polarizer and analyser are arranged to rotate about the microscope axis, as is also the stage on which the slide rests.

In more advanced instruments, especially those used for geological work, several mechanical modifications are made in order to permit the introduction of certain optical adjuncts for carrying out particular tests on such specimens—for example, provision must be made in the side of the body tube for inserting such things as a Bertrand lens or retardation plate (the purposes of which are described later). Also recent developments on some instruments tend towards a fixed stage and a synchronized rotation of polarizer and analyser (together with webbed eyepiece) in order to obviate delicate centring adjustments of the object—such a scheme is exemplified in the Dick model of polarization microscope.

The optical system, then, for a polarization microscope becomes as depicted in fig. 81. P is the polarizer (usually taking the form of a Nicol prism) situated immediately beneath the condenser C, and which can be rotated about a vertical axis. The analyser—also a Nicol prism—is generally arranged behind the objective at some such position as A_1: it can be moved out of the beam when desired by a slide in which it is fitted; usually, it is not rotatable, the "crossed" or "parallel" Nicol effect being produced by rotation of the polarizer. Alternatively, a second analyser, A_2, situated over the eyepiece, is frequently fitted, for use in certain cases when it is not possible to use the analyser in position A_1; for example, when using a retardation wedge (which of necessity must be used in the focal plane of the eyepiece and between polarizer and analyser) or when using the system for examinations in " convergent " light (described later). This analyser (which is rotatable) can also be swung conveniently out of position when not required. Slots in the side of the metal-work at S_1 and S_2 allow of the interpositioning of a quarter-wave plate and retardation wedge respectively when desired.

For a fuller description and a physical study of the polarization effects rendered with these accessories, the reader is referred to *Applied Optics*, Martin, Vol. I, pp. 202–221, and *Modern*

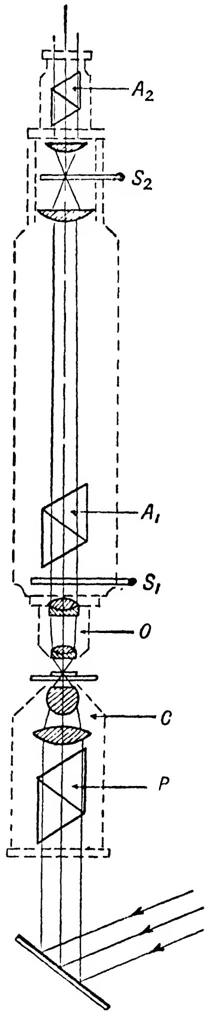


Fig. 81.—Optical System of a Polarization Microscope.

Microscopy, Cross and Cole, pp. 168–185. Suffice it to say here that if a thin wedge of a uniaxial crystal (e.g. quartz) —cut with its surfaces parallel to the optic axis—is placed between crossed Nicols and inclined at 45° to the vibration direction of the polarizer a series of interference bands will appear, the colours of which are dependent on the retardation between the two beams of the bi-refracting crystal when so arranged. Starting from black, when the thickness of the wedge is zero, bluish-grey tints are the first to appear followed by yellow, orange, red, &c., for positions of greater retardation; a study of the order of appearance of these colours for known retardations has been made by Quincke, and this is given below (Table VII). It will be realized, therefore, that the *colour* is a valuable indication of the relative retardation. If such a wedge be placed in position S_2 (fig. 81), and the specimen on the stage be rotated so that its "fast" vibration direction (explained later) coincides with the "fast" direction of the wedge, the order of the colour will be raised, but if arranged "fast" on "slow" the order will be lowered, and by recognition of the colours seen before and after super-position of the specimen, the amount of retardation may be determined.

With a knowledge of the retardation thus obtained and by knowing the thickness of the specimen under observation, the bi-refracting power or bi-refringence of a crystal may be obtained. The difference of the two refractive indices $(n_1 - n_2)$ of the crystal is known as its bi-refringence; the latter is related to the retardation and thickness by the expression $R = t(n_1 - n_2)$. As minerals are often tabulated in the order of their bi-refracting power (see p. 373, *Manual of Petrographic Methods*, Johannsen) the identification of the specimen is thus rendered possible. The chart of Michel-Levy (p. 371 of above work), which shows the colours visible for different thicknesses of crystal plates of varying bi-refringencies, is also a useful aid in such work.

TABLE VII.—COLOUR SCALE

(Adapted from Quincke's Table)

Retardation for "D" line (microns).	Interference Colours between crossed Nicols.	Order.
0·00	Black	FIRST.
·04	Iron-grey	
·097	Lavender-grey	
·158	Greyish-blue	
·218	Clearer-grey	
·234	Greenish-white	
·259	White	
·267	Yellowish-white	
·281	Straw yellow	
·306	Light yellow	
·332	Bright yellow	
·430	Brownish-yellow	
·505	Reddish-orange	
·536	Red	
·551	Deep red	
0·565	Purple	SECOND.
·575	Violet (the "sensitive violet")	
·589	Indigo	
·664	Blue (sky-blue)	
·728	Greenish-blue	
·826	Light green	
·850	Yellow green	
·910	Yellow	
·948	Orange	
1·101	Violet-red	
1·128	Bluish-violet	THIRD.
1·151	Indigo	
1·258	Greenish-blue	
1·334	Sea-green	
1·426	Greenish-yellow	
1·495	Flesh colour	
1·534	Carmine	
1·621	Dull purple	
1·652	Violet-grey	FOURTH.
1·682	Greyish-blue	
1·711	Dull sea-green	
1·744	Bluish-green	

"Fast" and "Slow" Directions

Mention has been made of "fast" and "slow" vibration directions. If a beam of plane-polarized light enters a double-refracting crystal perpendicular to the optic axis of the latter, the beam will, in general, be split up into two, each of which will travel with a different velocity through the crystal, and will also, therefore, have different refractive indices. Moreover, the vibrations in one beam will take place *parallel* to the optic axis and those of the other at *right angles* to the optic axis. Such materials may be looked upon as having "optical grain", and it can be shown that the beam in which the vibrations occur *along* the optic axis or "grain" either travels slower or faster than the beam which has its vibrations at right angles to the axis. Quite naturally, therefore, the "fast" direction denotes the direction of the vibrations in the beam which has the greater velocity (or lower refractive index) and vice versa for the "slow" direction; thus, for example, in quartz, the "fast" direction is perpendicular to the optic axis or "grain", whilst in calcite the "fast" direction is for the beam in which the vibrations occur "along the grain" or parallel to the axis.

The method of determining the "fast" or "slow" vibration directions of a specimen under observation on the microscope stage can be carried out by the use of an auxiliary microscope slide in which there are some small quartz crystals. These crystals must be of sufficient thinness to give a recognizable colour when placed with their axis at 45° to the vibration direction of the polarizer and examined with a Nicol set for extinction. It will be recalled that the "fast" direction of a quartz crystal is perpendicular to its optic axis, and therefore at right angles to the long edges of a particular crystal as seen in the microscope. If then the specimen is superimposed on the quartz crystal and rotated, a position will be found when the order

of the colour first seen is raised or lowered by a maximum amount; in the former case the " fast " direction of the specimen coincides with the " fast " of the crystal, and in the latter " slow " on " fast " will be indicated. In this way the fast and slow vibration directions of the specimen may be obtained.

Quarter-wave Plate

It will be clear that the foregoing method is somewhat limited, as it could only be used for low-power work (say $\frac{2}{3}$ in. objective or lower), so that for other cases a device known as a quarter-wave plate is employed for determining " fast " and " slow " directions. This consists of a cleavage plate of mica, the thickness (0·032 mm. approx.) of which is such that sodium light in passing through it along one of the two vibration directions possible in a double-refracting crystal, emerges with one element of the vibration retarded a quarter of a period behind the other—this results in circularly polarized light. The " fast " and " slow " directions should be marked on it (if not, they can be determined by the method described immediately above), and by inserting such a plate in the microscope as at S_1, fig. 81, and by rotation of the specimen under observation, the " fast " and " slow " directions of the latter may be differentiated; for " fast " on " fast " will give a rise in the colour scale, and " fast " on " slow " will lower the colour in the scale.

Examination in " Convergent Light "

One of the chief values in the use of so-called " convergent light " with the polarizing microscope is that it enables us to distinguish uni-axial from bi-axial crystals.

The term " convergent light " refers to *converging bundles of sets of (polarized) parallel rays* passing through the specimen, rather than a highly convergent cone of rays as might first be interpreted. In the examination of crystal sections by

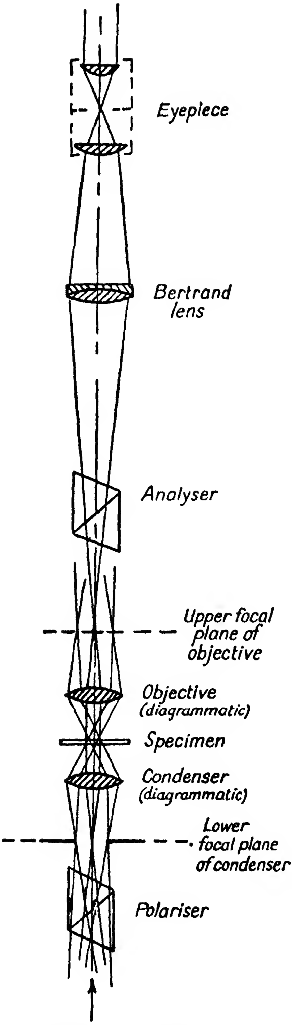


Fig. 82.—Arrangement of Optical Parts in a Polarization Microscope when used for "Convergent Light" Tests.

"convergent light" in the polarizing microscope, the effective light (already polarized) may be looked upon as originating in points in the lower focal plane of the condenser. This light passes through the crystal under observation *in parallel bundles at different inclinations* to the axis of the microscope, which are brought to a focus in the upper focal-plane of the objective, where the polarization effects can be examined. For this purpose an auxiliary lens, known as a Bertrand lens, is introduced into the microscope tube at the position indicated in fig. 82, which, together with the eyepiece, produces a low-power microscope system for observation of the upper focal-plane of the objective. This will be clear from the ray-diagram given in the figure, as will also the passage of parallel bundles of differently inclined rays through the specimen. With the microscope arranged in this way, it is possible to observe the interference effects produced by the specimen, for instance, the characteristic "ring and brush" figure of a uni-axial crystal or the "two eyes" appearance of a bi-axial.

Further information on this subject will be found in the following references:

REFERENCES

Evans, *Proc. Geologists' Assoc.*, Vol. XXI, Part 2, 1909.
Johannsen, *Manual of Petrographic Methods*, 1918.
Weinschenk, *Anleitung zum Gebrauch des Polarisationsmikroskops*, 1906.

What has been said in this section has been applied chiefly to geological or mineralogical work, but the use of the polarizing microscope in biological work does not appear to have received the attention it deserves. The differentiation and increased visibility in biological specimens rendered by the application of polarized light commends a study which might prove extraordinarily fruitful.

CHAPTER XII

Ultra-violet Microscopy

It will be recalled from previous pages that a microscope objective of given numerical aperture, when used with light of given wave-length, has a definitely limited resolving power. This resolving power, which may be represented by the least distance between two objects that the lens will reveal as two separate images, can be expressed by the relation $\frac{0{\cdot}5\lambda}{\text{N.A.}}$, where λ is the wave-length of the light concerned, and N.A. the numerical aperture of the lens. Such an expression tells us that if it is desired to obtain greater resolution or to see greater detail in an object there are two lines of approach open to us in order to attain this end; one of these is to make the numerical aperture of the lens system as large as possible, and the other to decrease the wave-length of the light with which it is used. The numerical aperture of the microscope objective has, for some time past now, practically reached

its physical limit—the N.A. of the highest-power immersion objectives being 1·40, although objectives of 1·65 N.A., used with an immersion medium of very high refractive index, are an experimental possibility—and therefore the reduction in wave-length of the light was considered the only hope of increasing the resolving power. The shortest wave-length of light of the visible spectrum which it is possible to use with the microscope in conjunction with the eye is approximately 450$\mu\mu$ (blue-violet) [although for comfortable work it is desirable to employ a longer wave-length at say 490$\mu\mu$ (blue-green), or even 540$\mu\mu$ (green)], thus it becomes necessary to employ ultra-violet light in order to reduce the value of λ, and as the eye is no longer sensitive to such radiations the photographic plate has to be substituted.

These facts were, of course, realized a great many years ago, and therefore it was not altogether surprising to find that thirty years ago attempts were made to produce a microscope for use with a shorter wave-length radiation. Such an instrument was designed by Köhler together with von Rohr, and several entire equipments of the apparatus were made by the firm of Zeiss; but strangely enough, although this instrument was made available at that date, comparatively little appears to have been done with it, and the War (1914–18) put an end to progress.

Within recent years, however, the subject has again been taken up, and definite advantageous results have been obtained with the " ultra-violet microscope ".

One of the first requirements in this work is that the whole optical system of the microscope must be made of some material which will freely transmit the ultra-violet region of the spectrum. When it is remembered that the lenses of the condenser, objective, and eyepiece, also the slide and cover-glass, must be of this material, it will be seen that the total thickness through which the radiation will have to pass may be as much as from ten to fifteen millimetres. On account of this, it is quite impossible to use

glass for the optical parts, as its transmission (in this thickness) is practically nil below wave-length $370\mu\mu$. The only substance which has yet proved satisfactory in this respect and also from an optical point of view is quartz (both in its natural and fused varieties); this can be used down to $200\mu\mu$ without fear of much opacity. There are possibilities in the use of fluorite on account of its transparency to even shorter wave-lengths, but its inherent properties tend to preclude its use. Up to the present, therefore, quartz (in both its forms) has been used for the optical system of the microscope; fused quartz for the objective, condenser, and cover-glass, and crystalline quartz for the eyepiece lenses and object slide. As it has not yet been found possible to combine a suitable substance with fused quartz for the purpose of achromatizing high power ultra-violet objectives, it is necessary to compute the latter for one wave-length only, and hence this series of lenses is known as "monochromats". A typical set of these objectives is given in the table below.

ULTRA-VIOLET ($\lambda=0{\cdot}275\mu$) MICROSCOPE OBJECTIVES (ZEISS)

Focus in MM.	6·0 (dry)	2·5 (immersion)	1·7 (immersion)
N.A.	0·35	0·85	1·25

The next requirement is that the illuminant for the microscope shall provide light which is strictly monochromatic and of the desired wave-length in the ultra-violet region for which the objective is designed. For this purpose a spark discharge is employed as the source, and the light from this is passed through a monochromator (see fig. 84), the optical components of which are of quartz (crystalline); one spectrum line thus formed is brought to a focus in the

plane of the condenser aperture. The ultra-violet region is rendered visible by means of the fluorescence of a piece of barium platino-cyanide screen or uranium glass held in the spectrum, and the desired line (to be used) can thus be brought on to the condenser. The electrical conditions for producing the spark are illustrated in fig. 83 and consist of a supply of alternating current (the periodicity of which is of no great importance) which is stepped-up by means of a transformer to a secondary potential of about 5000 volts for sparking across a 3 mm. gap. A condenser, the capacity of which is of the order of 0·0075 microfarads, is arranged in parallel with the spark.

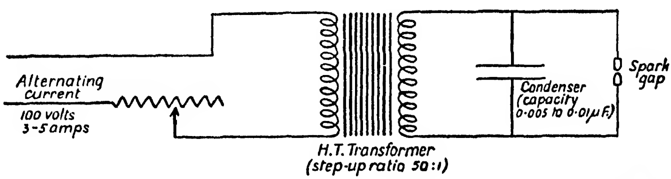


Fig. 83.—Electrical Apparatus (diagrammatic) in the Production of the Spark for Ultra-Violet Microscopy

Up to the present time, most of the work in ultra-violet microscopy has been carried out with a wave-length of $275\mu\mu$, that of a prominent line in the spark spectrum of cadmium, and therefore the electrodes at the spark gap in the above arrangement are of this metal. Further information on sources of illumination for this work is contained in a paper* referred to below.

Considerations of the depth of focus of the ultra-violet objectives will show that the mechanical requirements of this microscope are of a much higher order than the instrument used for visual work; especially is this so in the case of the " fine adjustment ". The depth of focus, it will be seen from p. 39, is directly proportional to the wave-length of the light employed, and if the former be determined for

* Johnson, *Phys. Soc. Proc.*, 1930, Vol. XLIII, Pt. 1, No. 236.

the three named lenses on p. 101, it will be found that the values are 0·0022 mm., 0·00052 mm., and 0·0002 mm. respectively. Thus the immersion monochromat objective has a depth of focus of 0·2 microns, but it is necessary in practice to displace the lens by only half this amount, and therefore errors in the mechanism employed for the translation of the objective (i.e. for focussing purposes) should not exceed 0·10 microns, or four millionths of an inch.

Such a mechanical device is, of course, rather difficult to make, but nevertheless, some have been made (notably in the Beck-Barnard instrument and also in a device* introduced by the authors) which do function satisfactorily within the stringent limits required of them.

The question of immersion fluid for use with the quartz objectives has presented some difficulty, on account of the apparent scarcity of liquids which will transmit the radiation at $275\mu\mu$ (or lower) and at the same time have a refractive index approximately near to that of fused quartz for this wave-length, namely, $n_{275} = 1{\cdot}4961$. Glycerin and water, mixed in right proportion to give the desired refractive index, has hitherto been employed as an immersion fluid; but under ordinary atmospheric conditions such a mixture is strongly hygroscopic, and with the consequent resulting change in refractive index an alteration in focussing of the object will take place. (Facts bearing on these statements, together with the way in which a non-hygroscopic immersion fluid may be made, are given in a paper by the writers referred to on p. 107, ref. 2.)

By making up a solution of cane-sugar ($n_D = 1{\cdot}4516$) and mixing this with a glycerin solution ($n_D = 1{\cdot}4530$) in the necessary proportions, a non-hygroscopic and non-evaporating fluid can be obtained. This will give a stable liquid of the correct refractive index for the condition of " homogeneous immersion " for a fused quartz objective used with wave-length $275\mu\mu$.

* Martin and Johnson, *Journ. Scien. Instrs.*, Vol. VII, No. 1, 1930.

The general arrangement of the apparatus necessary for ultra-violet microscopy is shown diagrammatically in fig. 84, which is a plan view of the equipment. The microscope is arranged in a horizontal position, and the light for *visual* observation is brought into the condenser from the source S_1 via the right-angled prism; the latter is conveniently swung out of position when the ultra-violet illumination is to be used. The radiation from the spark S_2 passes through the quartz components (forming the monochromator) and the spectrum thus formed is focussed in the plane of the condenser; by rotation of the monochromator the desired line is brought into the microscope. The ratio of the focal lengths of lens L_2 to L_1 is such that the enlarged image of the slit is sufficient to wholly fill the aperture of the condenser. The lens L_2 acts as the effective radiant and this is focussed in the plane of the object by adjustment of the condenser; an iris diaphragm I serves to control the size of the source. The remaining parts of the apparatus, namely, the camera and the microscope, do not call for particular mention, except, of course, that the microscope stand (together with fine adjustment) is of special design, and that the objective and eyepiece are of quartz and computed especially for the work, but these points have already been referred to.

The procedure adopted when using the apparatus for transmitted light may be divided into two methods; one of these (devised by Barnard) is to focus the object with a visual objective employing illuminating device S_1 and to record the fine adjustment reading; then to remove the glass lens and put in its place the quartz objective—for this a special type of mount is employed—set the fine adjustment to a predetermined correction-setting, and take three or four photographs, using the ultra-violet illumination with a change in focus of the microscope of $0{\cdot}1\mu$. Alternatively, a small piece of material (opaque to the radiation being employed, e.g. a minute piece of carbon or gold leaf) may be placed on the underside of the cover-glass before the latter is placed

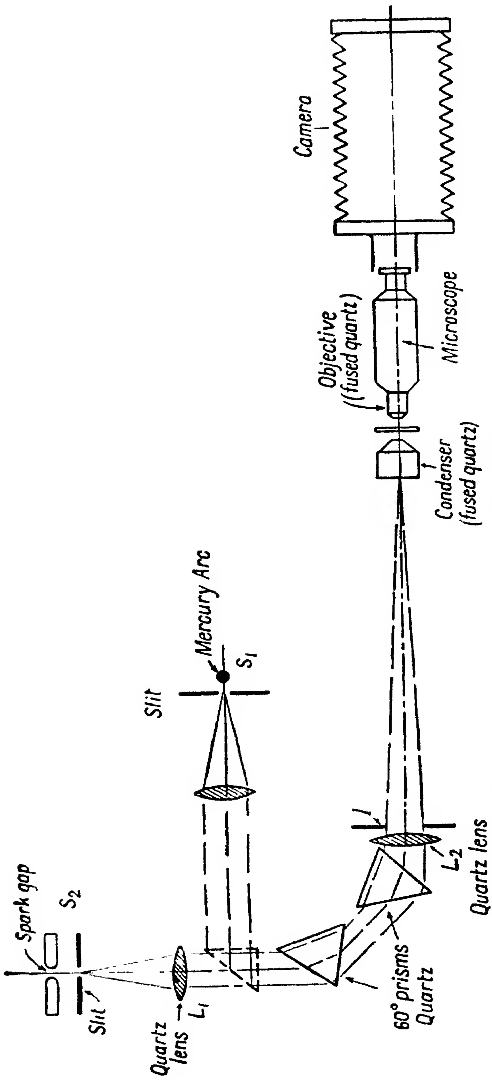


Fig. 84.—Arrangement of Apparatus for Ultra-Violet Microscopy

over the object and sealed down. This will enable the plane of the object to be focussed with ultra-violet illumination directly, by the employment of a fluorescent eyepiece placed behind the microscope with the quartz objective in position. For although the luminosity incurred when using the fluorescent eyepiece is so low that it is impossible to see a " transparent " type of object, a small speck of carbon or gold will appear very black in the field and will serve as a reference

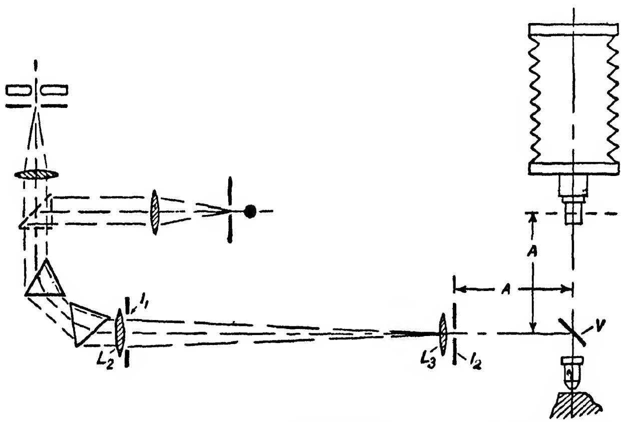


Fig. 85.—Apparatus for Ultra-Violet Metallography (diagrammatic)

mark on which to focus, and then a series of (say) four photographs is taken as before, when it will be found that a precisely focussed image on the plate will be obtained.

This latter scheme has the advantage of eliminating any possible error in change of focus of the object or its lack of registration due to the fact of removing one objective and replacing another as is done in the first method, although it must be mentioned that in spite of the change-over of objectives appearing an inadvisable system to adopt, the principle seems to operate satisfactorily in practice.

The arrangement of the optical system for ultra-violet metallography is shown in fig. 85.

As is usual, a " vertical illuminator " V is placed behind the objective, but in this case the illuminator plate must not be of glass, as this would not transmit the ultra-violet light on its return from the specimen through the objective to the eyepiece and camera. Either a quartz plate must be used or preferably a methylated collodion film, which serves admirably—(for method of preparation and mounting of latter see paper of reference No. 2 below). An iris diaphragm I_2, situated at the long conjugate point of the objective, controls the area of the illuminated portion of the object; whilst the lens L_3 forms an image of the iris I_1 on the back lens of the microscope objective, the aperture of which is therefore controllable. The other parts of the apparatus are the same as for " transmitted light " conditions already described, although it should be mentioned that in fig. 85 an image of the desired spectrum line is formed on the lens L_3.

It must be realized that the subject of ultra-violet microscopy is still in its infancy, and that the instrument has yet only been used by a very few people, but nevertheless the results which have been obtained are distinctly promising, and moreover it has been shown that the hoped for increased resolving power has actually been attained. The frontispiece shows comparisons in visual and ultra-violet illumination of a metal specimen and illustrates the increase in resolution when the shorter wave-length is employed. When this recent addition to the technique of microscopy has been exploited, and the instrument used by a larger number of workers, no doubt valuable information will accrue from the use of this new tool.

REFERENCES

Köhler, *Zeitschr. f. wiss. Mikr.*, 3, 129–165 and 273–304, 1904.
Martin & Johnson, *Jour. Scien. Instr.*, Vol. V, No. 11 and 12, 1928.
Johnson, *Proc. Phys. Soc.*, Vol. 42, Part 1, No. 231, 1929.
Lucas, Reprint B. 200, Bell Telephone Laboratories, U.S.A., 1926.
Barnard, *Journ. Roy. Micr. Soc.*, Vol. XLIX, Part 2, 1929.
Johnson, *Proc. Phys. Soc.*, Vol. XLIII, Part 1, No. 236, 1930.

CHAPTER XIII

On the Interpretation of the Image in the Microscope

Imagine that the object consists of a pattern drawn on a slide with opaque ink; it has no appreciable thickness, and the slide can be held at right angles to the microscope axis. Confining our attention to the middle of the field, we vary the focus until the image shows a pattern of maximum sharpness. There is then more or less *geometrical similarity* between the central dark parts of the image and the corresponding opaque portions of the slide.

Towards the margin of the image, the sharpness of the border between bright and dark portions is perhaps diminished. Does this indicate a corresponding "fuzziness" in the object? We test the point by a small alteration in the focus. If we can thus sharpen the image the fuzziness is attributed to an out-of-focus effect; if not, there may be a lack of sharpness in the object. Without such a test, a single picture, such as a photomicrograph, is very hard to interpret.

Let us now take an eyepiece of much higher power, and again examine the image. The boundary between dark and light regions is now less definite. It cannot be made completely sharp at any focus. If we can imagine that any bright area of the object is divided up into an infinitely great number of bright points, then we know that the image of each of these bright points is a *disc* of finite magnitude. Each disc is brightest in the centre, fading toward the circumference. Suppose that the object contains a sharply pointed spear of dark material such as (*a*), fig. 86, then the bright elements all round the edge, being imaged as discs, will soften the boundary of the image, and will make an apparent rounding-off of the point (*b*). In order to obtain a sharper image, the

discs must be made smaller. This can be effected by using an objective of greater numerical aperture, or by using light of shorter wave-length. Also the numerical aperture of the illuminating beam must be as large as possible consistent with the avoidance of glare; this usually calls for a numerical aperture somewhat smaller than that of the objective itself.

Improvement in such directions will secure a "sharper" image, and the finer details then have an increasing geometrical similarity to the object. If the system is well corrected optically, the out-of-focus appearances will be very similar on each side of the best focus, especially if a green filter is used to mask the effects of residual chromatic aberration, and we need not often be in doubt as to the setting for the true focus with such a simple type of object as described above. On the other hand, if the system suffers from spherical aberration, then we may sometimes be in some doubt as to the best focus, the "picture" showing perhaps a fringe of light within an apparently sharply focussed edge. But the marked dissimilarity of the out-of-focus appearances on each side will provide the warning, and cause us to pay the necessary attention to tube length or cover-glass thickness.

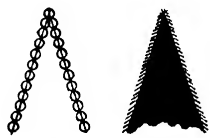

(a) (b)

Fig. 86

The majority of microscopic "objects" are not of such a simple character. They have a definite thickness and may exhibit all degrees of opacity and colouration; in many cases they have *no* opacity or colouration of an appreciable amount, but manifest their presence by the difference in refractive index between themselves and the surrounding medium. This difference causes deviation and reflection of the light rays at the boundary.

Let us first of all consider an object consisting of opaque material of finite thickness. An instructive example can be made by mounting some very small mercury globules in a slide, and examining the appearances in the image.

It is a common mistake to think that the picture in the microscope represents a plane section of the object taken in the plane of the focus conjugate to the image plane in the optical sense. If this were true we should be able to obtain a series of pictures of a mercury globule by changing the focus in successive steps with results like fig. 87. This is manifestly not the case; in fact, the appearances depend greatly on the reflection and diffraction of light around the globule, and the actual result is shown in a *photograph* (see fig. 88). At one stage, the appearance suggests a bead pierced by a central hole, an effect entirely due to the action of the light.

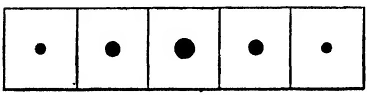


Fig. 87.—Expected Appearance (without consideration of Optical Phenomena) of a Mercury Globule at different focussing positions of the Microscope.

Another "artificial" object, but one which is representative of cases which frequently arise in practice, can be made by mounting in a slide a very small quantity of an emulsion containing small fat globules suspended in a watery medium. In this case, both refraction and internal and external reflection of the light at the surface of a globule (which usually has a higher refractive index than the surrounding medium) complicate the appearances. When the objective has its focus adjusted to the plane of the remote side of the globule, the appearance is of a disc with a faint dark ring near the boundary; on racking upwards the light concentrates into a very bright spot near the centre of the globule, surrounded by correspondingly dark rings.

Filaments of glass (glass wool) mounted in media of slightly lower refractive index (say glycerin and water) are also very instructive to observe. At one stage of the focus there will be the appearance of a thin light or dark border to the filament, very much suggesting a sheath of some other material surrounding the thread of glass. It is frequently the case that appearances of this kind are

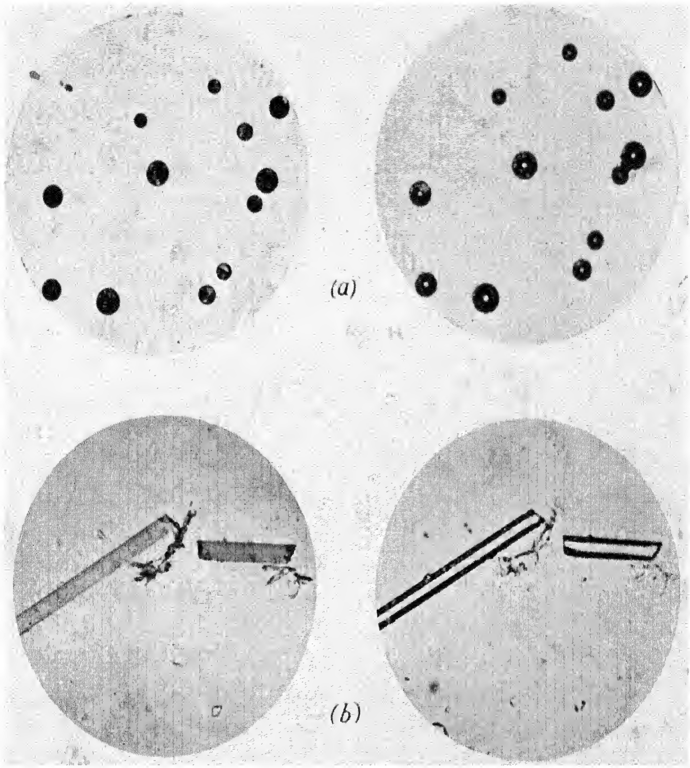


Fig. 88.—SPURIOUS EFFECTS DUE TO SLIGHT CHANGES IN FOCUS, SHOWING THE CARE NECESSARY IN INTERPRETING THE IMAGE

(*a*) Mercury globules mounted in cedar oil.
(*b*) Glass threads mounted in water.

Objective:—16 mm.
Numerical Aperture = 0·28
Magnification = 250 ×
Condenser:—$\frac{2}{3}$ aperture of objective
Wave-length of Light = 0·45μ.

mistaken for indications of actual physical structure: membranes surrounding bacterial cells, fine ducts or opaque threads in filaments, lamellæ in the substances of materials —such appearances may be purely optical effects.

Especially in the use of very oblique or dark-ground illumination, these optical effects are prominent. The borders of structures are sometimes apparently fringed by alternate dark and bright fringes which are purely diffraction phenomena; but in observing diatoms they have been mistaken for "ribs" or structural details.

It is not possible to give any special general rule for testing the "reality" of any structure which may be suspected, but certain tests may be carried out, and the following are suggested.

1. Doubtful striæ. Observe the object first with white light, next with homogeneous light, such as that of the mercury lamp, with a suitable green filter. If the phenomenon is a diffraction effect, its prominence will then be enhanced; several more fringes will usually be visible than with white light. Observations should also be made with yellow light and blue light alternately. If the striæ are due to diffraction they will be more closely spaced with blue light than with yellow, whereas if they are due to definite structure in the object their apparent spacing will be unchanged.

2. Observe the nature of the appearances with varying numerical aperture of the illumination; spurious effects are usually least prominent when working with a solid cone of the largest aperture which can be used without the introduction of too much glare.

3. If any particular structure is suspected, it may be possible to make an artificial object of analogous optical nature, as has been suggested above in the case of the filaments of glass wool, and observe it with the microscope, using a lower numerical aperture if the artificial object is on a larger scale than the actual one. Or it may be possible to find a natural object of known structure made on a larger scale. Thus

considerable light on the structure of the diatom " Pleurosigma angulatum " has been thrown by investigations of the coarser diatom " Triceratium favus ". The latter, when viewed by objectives of low N.A., presents an image very similar to that of the former, when the highest apertures are employed.

It is no part of the present discussion to deal with complex theoretical points, but a word may be added on the methods of seeking for evidence of regular periodic structure. Where such is suspected, as in a diatom, it is most readily detected by the use of a narrow beam of oblique light through the object, and is generally produced by the use of an eccentric small stop below the condenser. In some microscopes the stop can be moved radially by a small rack and pinion. The structure might be, for example, a group of serrations along a certain structure; then the displacement of the stop should be parallel to the line containing the elements of the serration; or it might be a group of parallel " ribs ", then the displacement of the stop would be perpendicular to the ribs. Care must always be taken to avoid misinterpreting diffraction effects under such conditions.

When using oblique light of narrow aperture it is sometimes helpful to observe the diffraction effects in the back focal plane of the objective. In the case of a feebly differentiated structure we may then see a bright patch of light corresponding to the image of the condenser stop: it will be apparently at the margin of the back aperture; there may also be a diffracted patch just visible at the opposite end of the diameter. In this case the presence of such a diffracted beam would provide evidence of the existence of a structure of calculable dimensions, even though the diffracted beam were too feeble to produce any noticeable effect in the actual image.

The action of regular structures in diffracting light must be remembered when colouration of an unaccountable nature appears in the microscope image, especially with dark-ground illumination where narrow annular beams of light may illumi-

nate the object. These beams are diffracted by the structure, and the angle of diffraction varies with the colour of the light; blue light of relatively short wave-length is deviated by diffraction less than red light of longer wave-length. White light is therefore dispersed by diffraction. It frequently happens that when a diatom such as " Amphipleura pellucida " is illluminated by a dark-ground condenser, the direct light does not enter the objective, and the diffracted light is so far deviated that the red and yellow parts also fail to enter. Hence the image is formed by the remaining light, and appears a bright blue-green.

In other cases characteristic colour fringes in parts of the object have to be attributed to the chromatic aberrations of the microscope system; they tend to appear in parts of an object which are not in perfect focus.

Again, colour may arise by the selective effects noticeable in the scattering of light by small particles which may be present in the medium of the object, even though they are too small to be distinguishable by ordinary methods. When the scattering particles are very small, the intensity of the scattered light varies inversely as the fourth power of the wave-length. The medium containing them therefore tends to appear blue by reflected or scattered light, and yellow or brown by transmitted light.

When objects are viewed by reflected light, using a suitable " vertical " or " ring " illuminator, colour may arise by reason of successive reflection at the surfaces of a film on the material; this colour may mask the characteristic colour of the underlying substances. Sir Herbert Jackson has found it possible in some cases to remove a great deal of the surface-reflected light by illumination with polarized light, using a ring illuminator. The image is then observed through a Nicol prism or other suitable analyser turned so as to extinguish the major portion of the surface-reflected light. Some of the light reflected at the lower layers is, however, depolarized, and is not extinguished by the analyser, so

that the characteristic appearances of the lower layers can be recognized.

For fuller details on the above subject reference may be made to the article "Microscope", by Sir Herbert Jackson and Dr. H. Moore, in the recent edition of the *Encyclopædia Britannica*.

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